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14. ABSTRACT Preparedness for acts of chemical terrorism and warfare require detection and response. Chemical testing will allow for diagnosis of exposed individuals. Acrylonitrile (AN) is a toxic industrial chemical produced in large quantities by the chemical industry and is acutely toxic. Our objective was to define the chemical signatures of AN-adducts in human blood following an exposure. In this project we have measured the second order rate constants for the reaction of AN with the most reactive sites in human blood in vitro. Human blood, red blood cell lysates and plasma were incubated, under pseudo first-order conditions, with 100mM AN at 37°C and the disappearance of glutathione (GSH) and the appearance of the AN-adducts of GSH, hemoglobin beta-Cys93 (Hb•C93-AN) and albumin Cys34 (AbC34-AN) were monitored. The second order rate constants in M-ls-1 were: disappearance of GSH in whole blood, 0.0806; appearance of GS-AN in whole blood, 0.0776, appearance of Hb•C93-AN in rbc lysate, 0.000722 and appearance of AbC34-AN in plasma, 0.224. The data indicate that the most reactive site for AN in human blood is Cys34 of albumin. This site reacts 2.8 times faster than GSH and 310 times faster than Hb•C93 and thus will serve as an excellent biomarker of AN exposure.					
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Introduction:

The twenty-first century warfighter will encounter the challenge of the potential use of toxic industrial chemicals (TICs) as chemical warfare agents. Preparedness for acts of chemical terrorism and warfare require detection and response to such attacks. Appropriate chemical testing will allow for the proper diagnosis and treatment of exposed individuals. The purpose of this project in the submitted proposal was to define the chemical signatures of the TICs acrylonitrile and acrolein in human blood, and potentially to devise rapid, high throughput screening technology to enable examination of large groups of individuals following a known or suspected exposure. Studies would be directed toward defining the patterns of chemical reactivity of blood components with these TICs in order to use these adducts as biomarkers of chemical exposure. Before these studies could begin, new mass spectrometry equipment was purchased, site preparation was completed and operators were trained in its use.

Body:

The original P.I. of this project was Dr. William M. Pierce, who served in this capacity from April 2008 through November 2009. Subsequently, Dr. Frederick W. Benz was approved as the P.I. and has served in that capacity for the final eight months of the project after Dr. Pierce became the interim Vice President for Research at the University of Louisville.

Our initial experiments were directed toward characterizing the MS spectra of the expected blood adducts of the two toxic industrial chemicals (TICs) under study, namely acrylonitrile (AN) and, in future studies, acrolein (AC). The most likely blood biomarkers of exposure to these TICs could be predicted based on their chemical reactivity with nucleophiles and specifically with the highly reactive cysteine residues in blood. Thus red blood cell glutathione (GSH), plasma albumin cysteine-34 (HSA-C34-AN) and red blood cell hemoglobin beta chain cysteine-93 (Hb β C93-AN) would be the expected targets.

Glutathione Adducts:

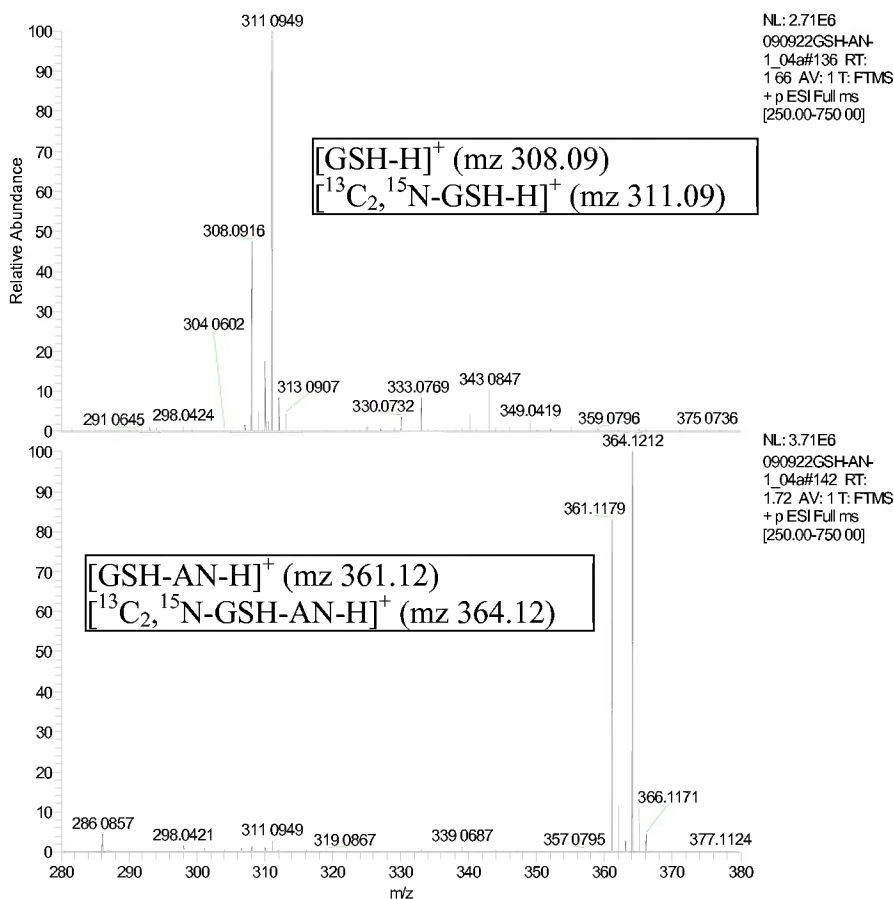
The tripeptide, γ -L-glutamyl-L-cysteinyl-glycine (glutathione, GSH), is the most important low molecular weight antioxidant synthesized in cells. It is synthesized by the sequential addition of cysteine to glutamate followed by the addition of glycine. The sulfhydryl group (–SH) of the cysteine is involved in reduction and conjugation reactions that are usually considered as the most important functions of GSH.

GSH is found in the cytosol of cells where it is in the range of 1–10 mM (1). In most cells the GSH concentration is about 1–2 mM, while in hepatocytes, which export GSH, the concentration can reach about 10 mM. Whole blood GSH is approximately 1mM, with virtually all of it inside the red cells.

The elimination of many xenobiotic compounds can be accomplished through conjugation with GSH, both non-enzymatically and enzymatically catalyzed by a glutathione-S transferase (GST). GSH is known to be involved in the elimination of many electrophiles such as HNE and also AN (2).

In order to monitor AN-alkylation of GSH in whole human blood, we synthesized the glutathione-acrylonitrile adduct (GSH-AN) and a stable isotope labeled analog $^{13}\text{C}_2, ^{15}\text{N}$ -GSH-AN, which would

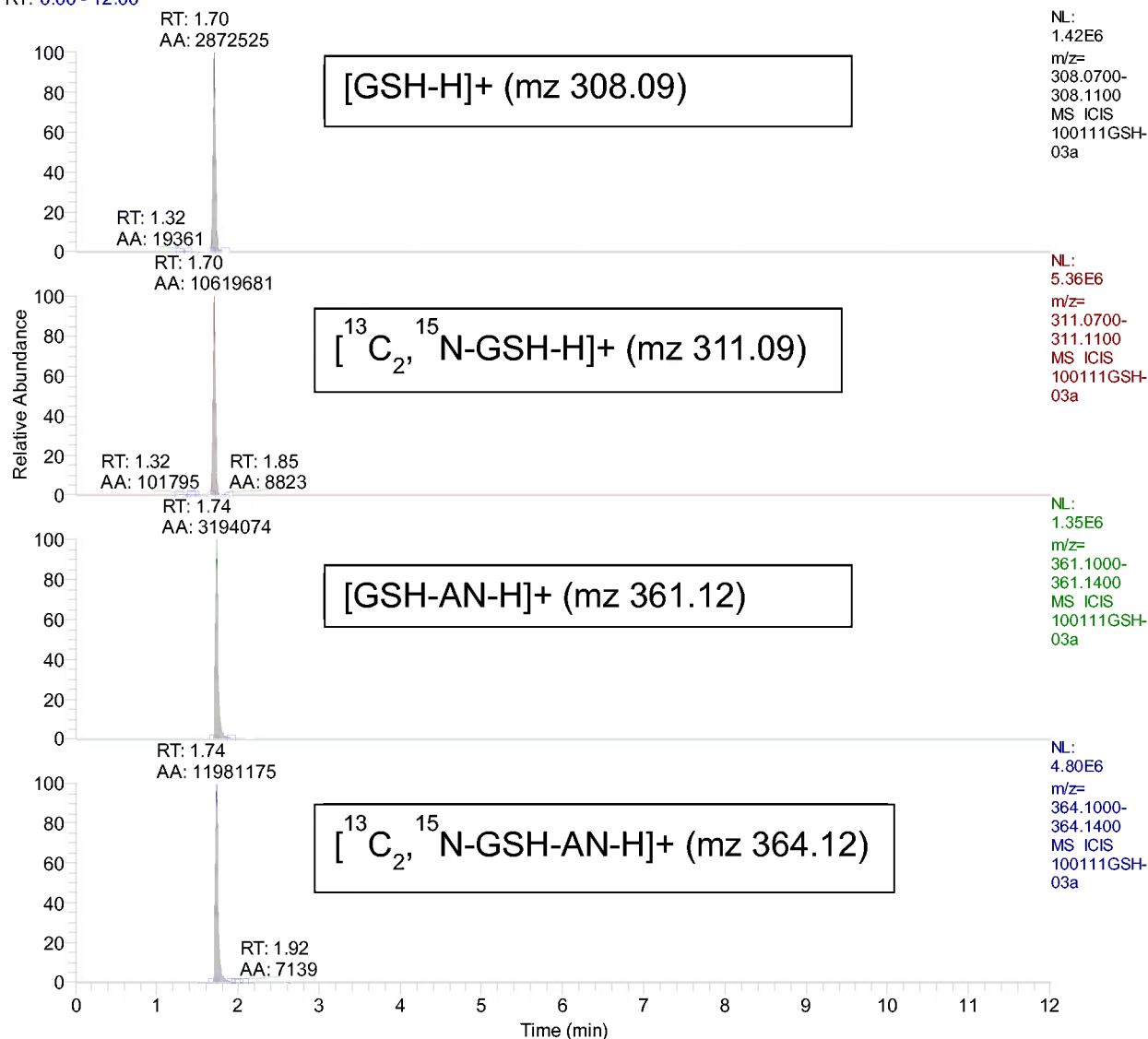
serve as an internal standard for quantification. The MS spectra of unmodified GSH and the GSH-AN adducts are illustrated below.



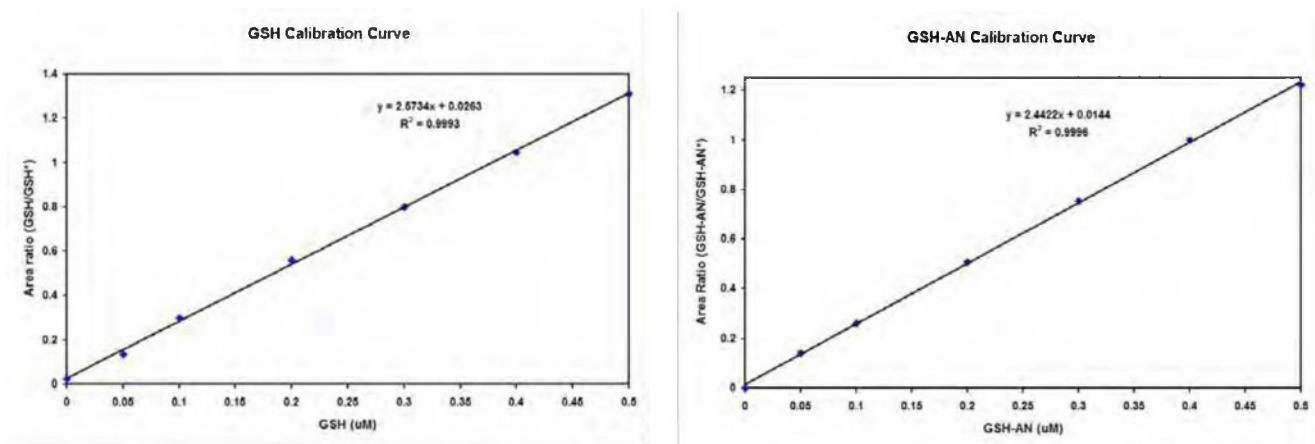
Top figure shows the MS spectrum of a mixture of unmodified glutathione [GSH-H]⁺ (m/z 308.09) and its isotope labeled internal standard (m/z 311.09). Bottom spectrum shows the mixture of the glutathione-acrylonitrile adduct [GSH-AN-H]⁺ (m/z 361.12) and its isotope labeled internal standard (m/z 364.12). MS/MS analysis of the [GSH-AN-H]⁺ precursor ion (m/z 361.12) yielded fragment ions at m/z 215.05 and m/z 232.08, while MS/MS analysis of the [¹³C₂, ¹⁵N-GSH-AN-H]⁺ precursor ion (m/z 364.12) yielded fragment ions at m/z 218.04 and m/z 235.08 all of which are consistent with the chemical structures of the AN adducts of GSH.

Standard solutions containing variable amounts of GSH-AN with a fixed amount of ¹³C₂, ¹⁵N-GSH-AN (GSH-AN*) as internal standard were prepared and analyzed by LC/MS and peak areas of ion chromatograms of molecular ions were used for quantification. A similar approach was utilized for quantification of unmodified GSH. A typical set of chromatograms collected for these analytes are illustrated below.

RT: 0.00 - 12.00



From this data, calibration curves could be constructed for the quantitative analysis of unmodified GSH and its AN-adduct.

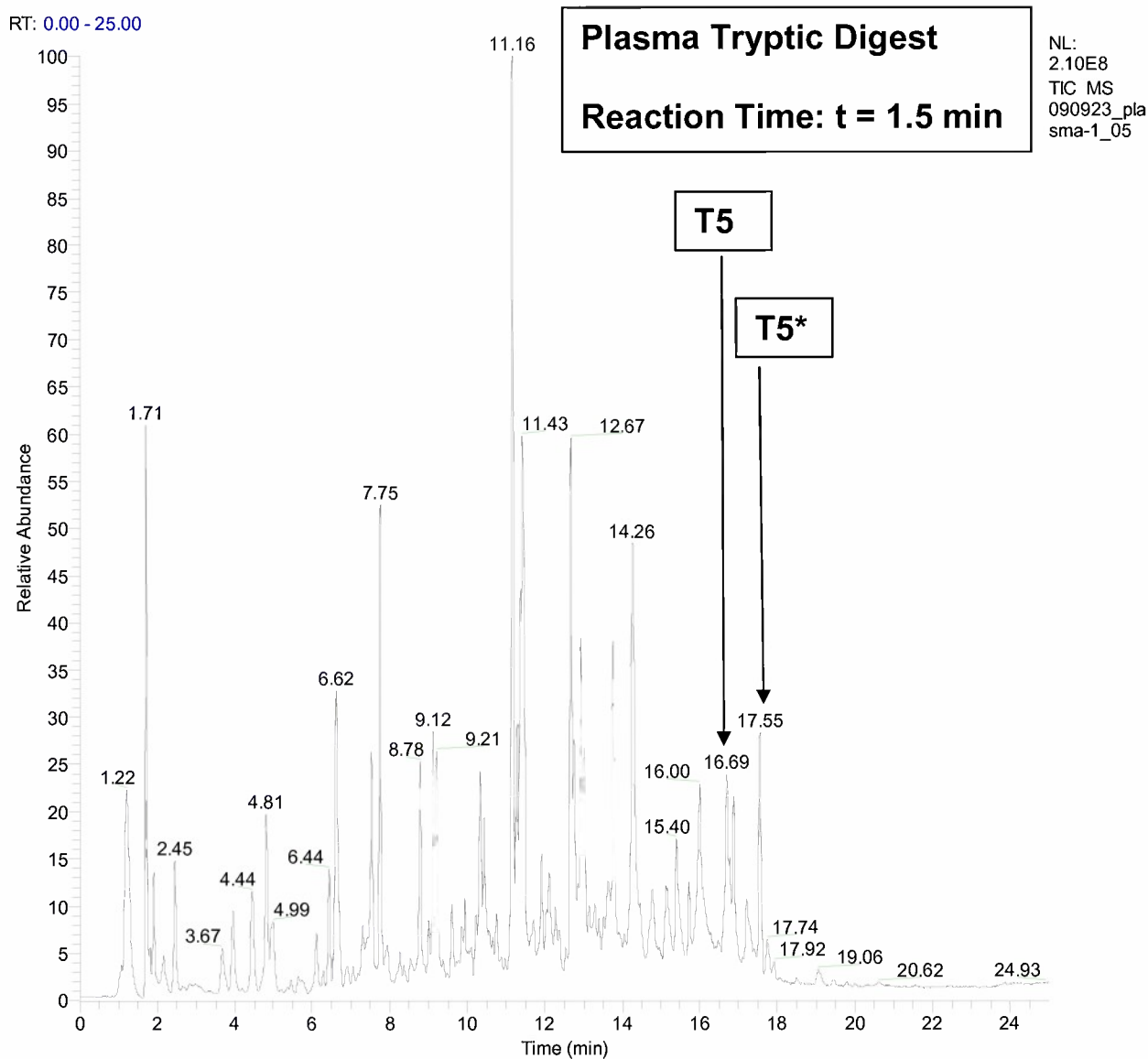


With this approach, we now had methods in hand to measure the depletion of whole human blood GSH and the formation of the GSH-AN adduct as biomarkers of AN exposure.

Albumin Adducts:

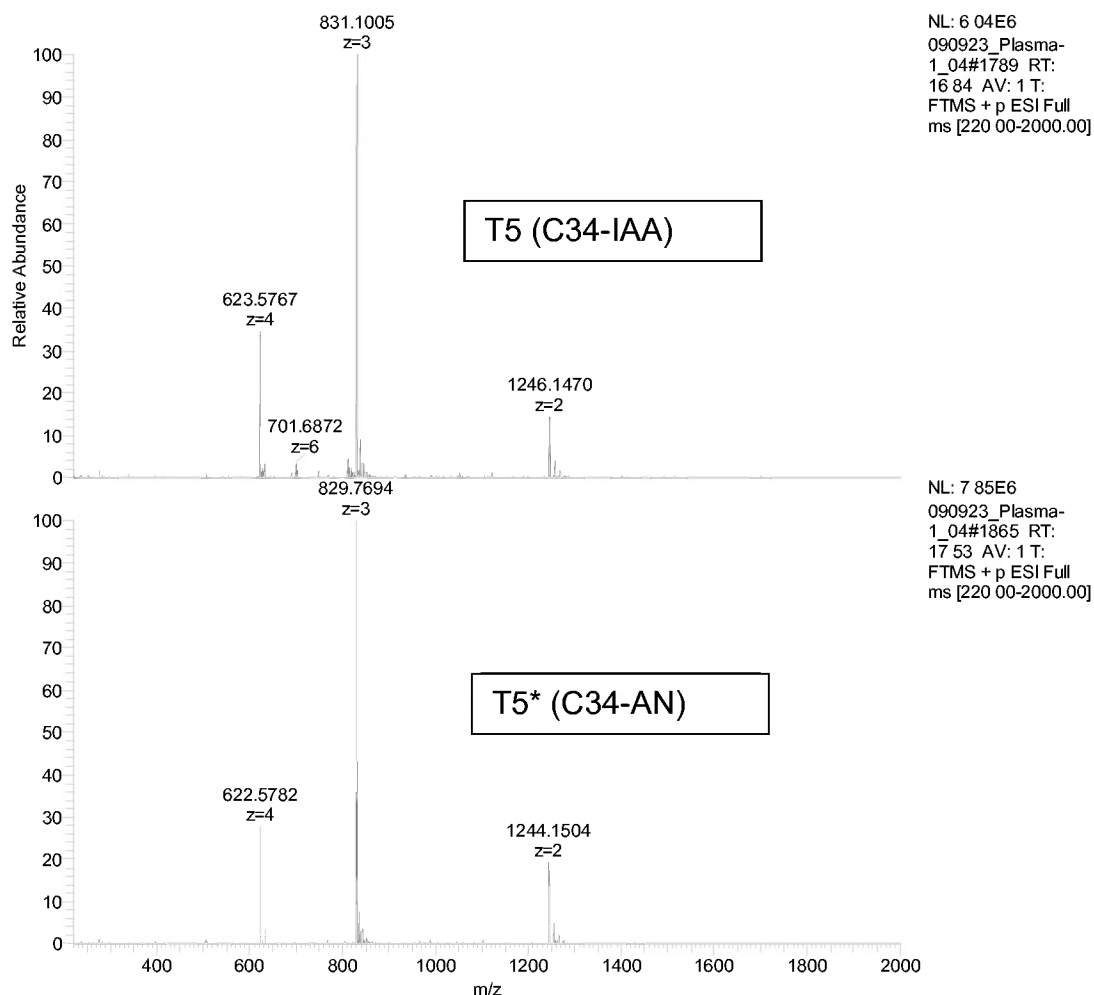
Human serum albumin (HSA) contains 35 cysteine residues of which 34 are paired as intramolecular disulfide bonds (3). The remaining cysteine, cysteine-34 (HSA-C34) is highly reactive with electrophiles (4). It also represents the largest fraction of free thiol in blood serum at about 0.6mM, although approximately 30% of C34 is known to be bound as a mixed disulfide with either cysteine or glutathione. Thus free C34 is a leading candidate to serve as a biomarker of AN exposure.

To monitor the reaction of AN with HSA-C34 the following method was developed. Plasma was reacted with AN and samples were taken at various times after starting the reaction and plunged into acetonitrile to stop the reaction and precipitate the plasma proteins. The plasma protein precipitate was subsequently reduced with dithiothreitol and the free cysteines were alkylated with iodoacetamide. The reduced and alkylated plasma proteins were then digested with trypsin and an aliquot of the tryptic digest was applied to a Hypersil Gold C18 reverse-phase column (50 x 2.1mm) and the peptides were eluted with an acetonitrile gradient containing 0.1% formic acid. A typical chromatogram of the plasma tryptic (T) peptides is shown below.

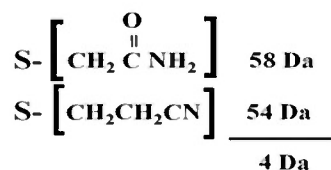


The peptide labeled T5*, eluting at 17.55 min, is the tryptic peptide of human serum albumin containing the C34-AN adduct. The peptide labeled T5 is the identical peptide containing C34 alkylated with iodoacetamide, indicating the fraction of albumin C34 that had not yet reacted with AN and thus was still free at 1.5 min into the reaction.

The mass spectra of these two peptides are illustrated below.

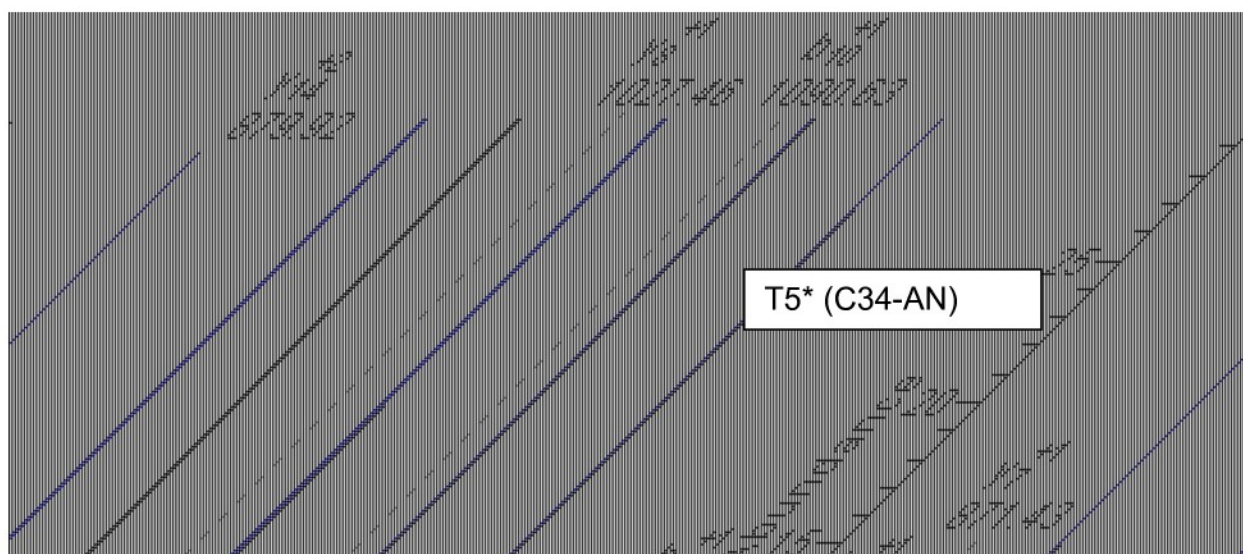
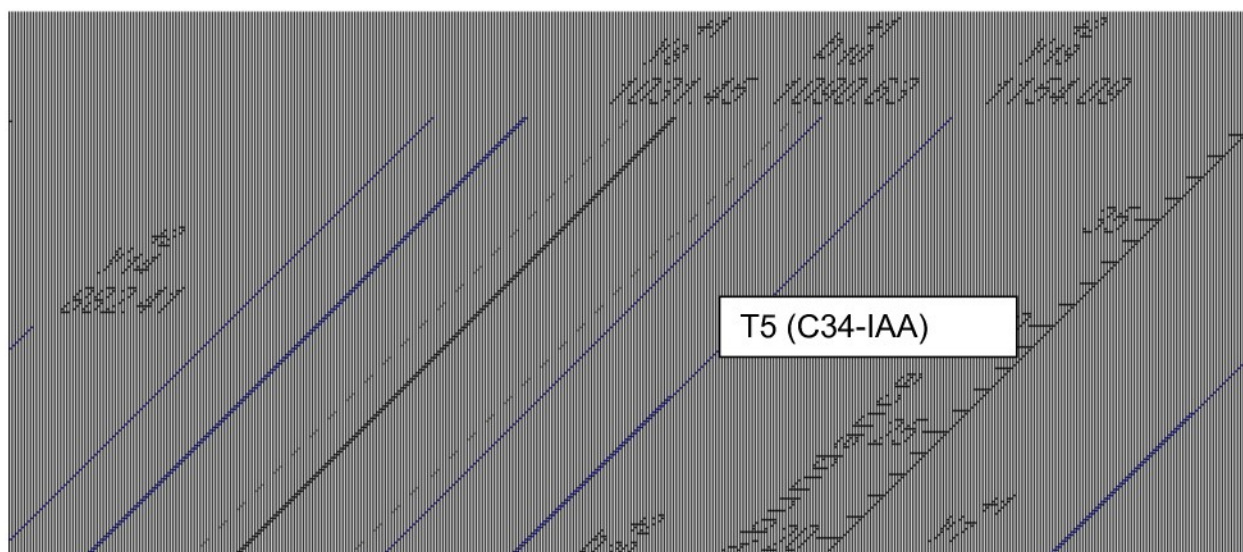


Notice the m/z for the triply charged most abundant isotope peak of the peptide with the AN-adduct is 829.7694, whereas the isotope peak of the identical peptide blocked with iodoacetamide is 831.1005. The m/z difference being 1.3311. This can be explained as follows.



As shown above, the expected mass shift for a cysteine residue that has reacted with AN versus that blocked by iodoacetamide is 4Da. However, this peptide carries a triple charge, thus the 4Da must be divided by 3 to get expected m/z difference of 1.33 as observed in the spectra above.

The identification of the above peptides as being the T5 tryptic peptide of HSA was done by analysis of the MS/MS spectra. A portion of these spectra are shown below and are consistent with the sequence of HSA T5 and also indicate the site of AN adduction on this peptide.



The sequence of HSA peptide T5 is shown below:



The site of the AN-adduct on this peptide is readily apparent from the 4Da shift, which begins at the amino acid site of adduction. Specifically, the y_7+1 ion in both T5 and T5* is at $m/z = 871.43$, whereas the y_8+1 ion in T5* ($m/z = 1027.48$) is shifted 4Da from the same ion in T5 ($m/z = 1031.45$), indicating that C34 is the site of the AN covalent adduct. This is confirmed by the b-ion series, where $b_{13}+1$ ion occurs at $m/z = 1459.83$ in both T5 and T5*, whereas the $b_{14}+1$ ion in T5* ($m/z = 1615.86$) is shifted 4Da from the equivalent ion in T5 ($m/z = 1619.86$).

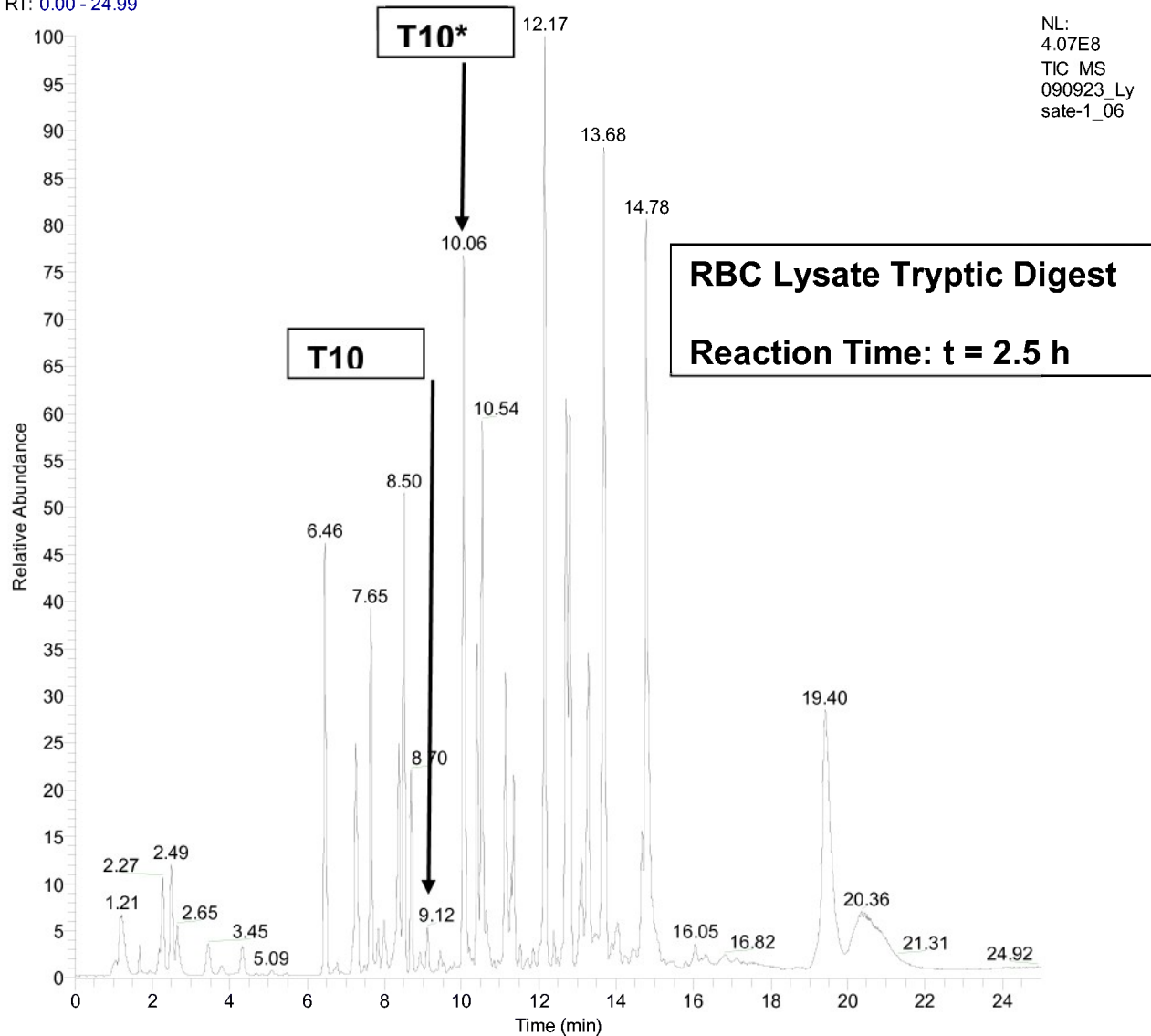
From this analysis, we now had a method in hand to monitor AN exposure by assessing the level of AN adduction of C34 in human serum albumin.

Hemoglobin (Hb) Adducts:

In addition to albumin, hemoglobin, also being abundant in blood, has long been used to determine chemical adduct levels (5). The benefits of Hb adduct determination as an internal dose monitor for exposure were first suggested in 1976 by Ehrenberg and co-workers (6). Originally, adducts to histidine residues in Hb were determined in blood samples from chemically-exposed persons. However, application of this methodology was hampered by the complicated and tedious methods for sample preparation. A major improvement was realized in 1986 by Tornqvist and co-workers with a modification of the Edman-degradation method that made reliable determination of Hb adducts to the N-terminal valine by GC-MS feasible (7). However, this methodology although sensitive and accurate is also relatively time-consuming and thus would not be suitable for triage purposes. In addition, hemoglobin contains a relatively reactive cysteine in position 93 of each of its two beta chains (Hb β C93), which is likely to be more reactive toward acrylonitrile than the N-terminal valine. Finally, high resolution and high sensitivity mass spectrometry instruments have recently been developed so that adducts on hemoglobin cysteine-93 can be assessed. For all of these reasons we sought to develop a method to monitor acrylonitrile adduction to hemoglobin for use as a biomarker of exposure.

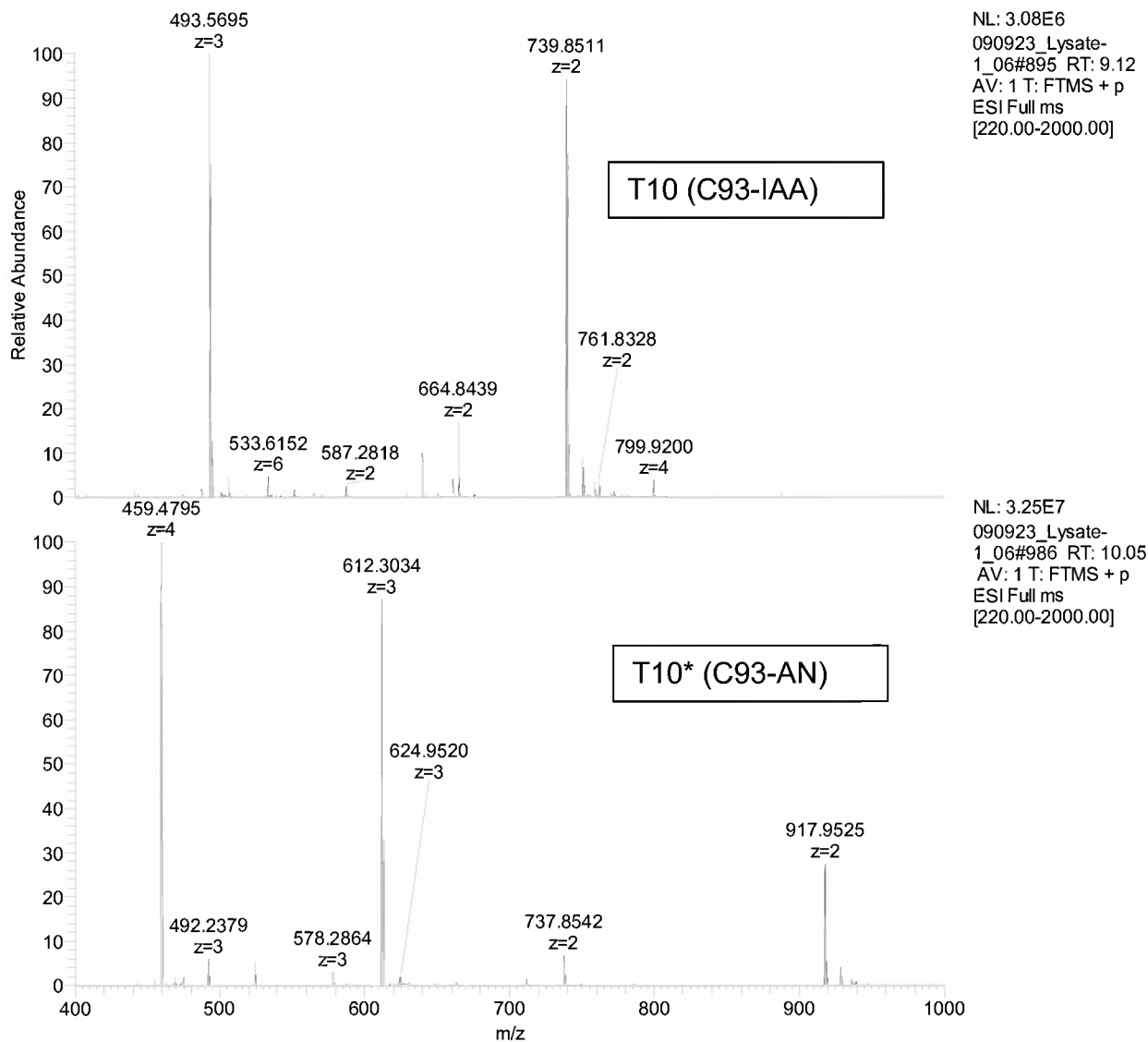
For the determination of the reaction of AN with β Cys-93 of hemoglobin, red blood cells were prepared from fresh human blood by centrifugation. The cells were washed twice with PBS and then lysed with two volumes of distilled water. The initial lysate was further diluted with distilled water and 0.27M ammonium bicarbonate to a final concentration of approximately 1mM in hemoglobin β Cys-93 and 50mM ammonium bicarbonate, pH 7.4. The final lysate was then incubated with AN and aliquots were removed at various times and plunged into acetone containing 0.1% HCl to precipitate the proteins and to stop the reaction. RBC proteins were pelleted by centrifugation, alkylated with iodoacetamide, digested with trypsin and the peptide digest was applied to a Hypersil Gold C18 reverse-phase column (50 x 2.1mm) and the peptides were eluted with an acetonitrile gradient containing 0.1% formic acid. A typical chromatogram of the RBC lysate tryptic (T) peptides is shown below.

RT: 0.00 - 24.99



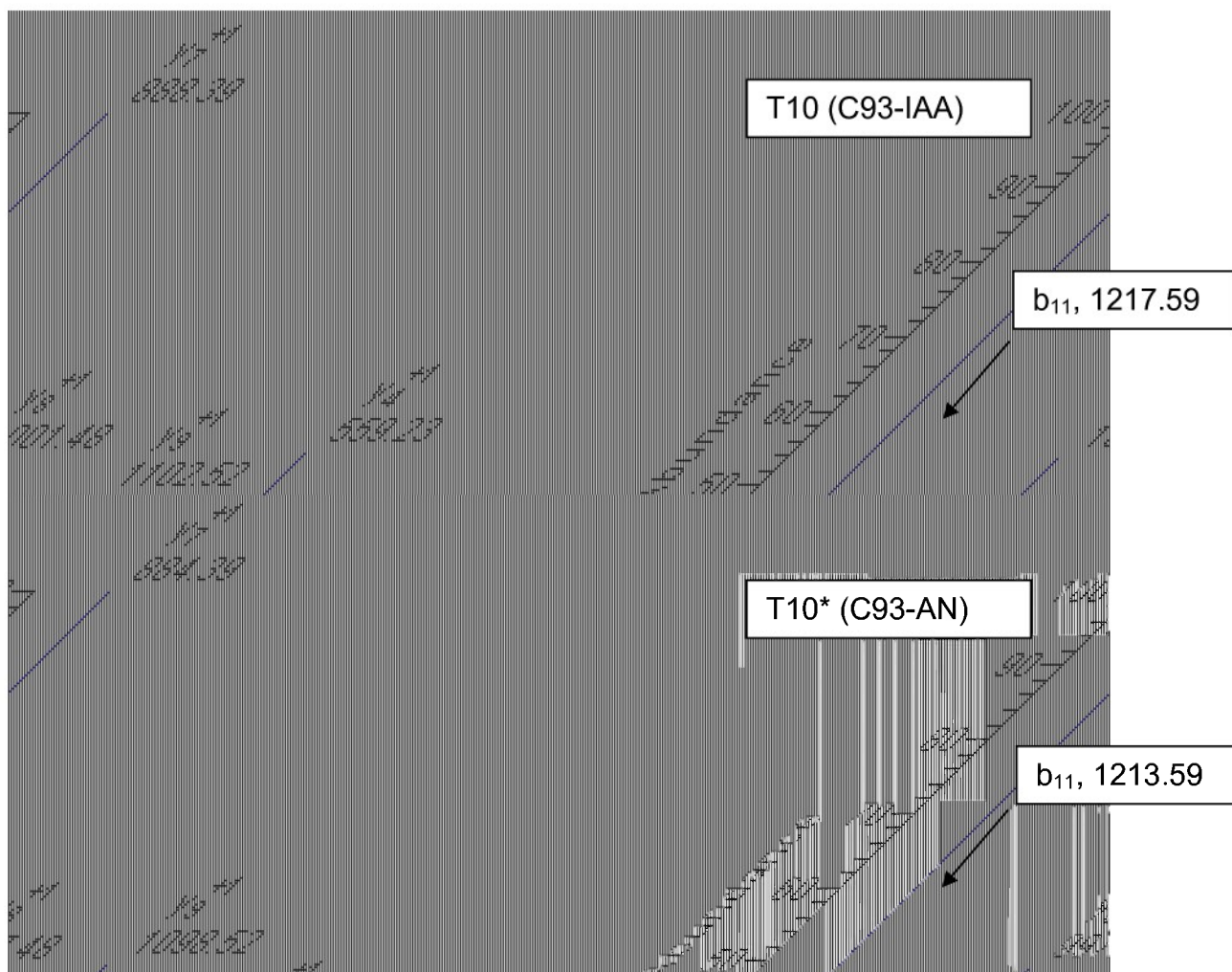
The peptide labeled T10*, eluting at 10.06 min, is the tryptic peptide of human hemoglobin containing the β -C93-AN adduct. The peptide labeled T10 is the identical peptide containing β -C93 alkylated with iodoacetamide, indicating the fraction of β -C93 that had not yet reacted with AN and thus was still free at 2.5h into the reaction.

The mass spectra of these two peptides are illustrated below.



In this instance, T10* containing β -C93-AN (m/z 737.8542, $z=2$ and m/z 492.2379, $z=3$) co-eluted with the peptide T6 containing α -chain residues 41-56, (m/z 917.95, $z=2$; m/z 612.30, $z=3$; and m/z 459.48, $z=4$).

The identification of the above peptides as being the T10 tryptic peptide of Hb β was done by analysis of the MS/MS spectra of these peptides as shown below.



The sequence of Hb β chain peptide T10 is shown below:

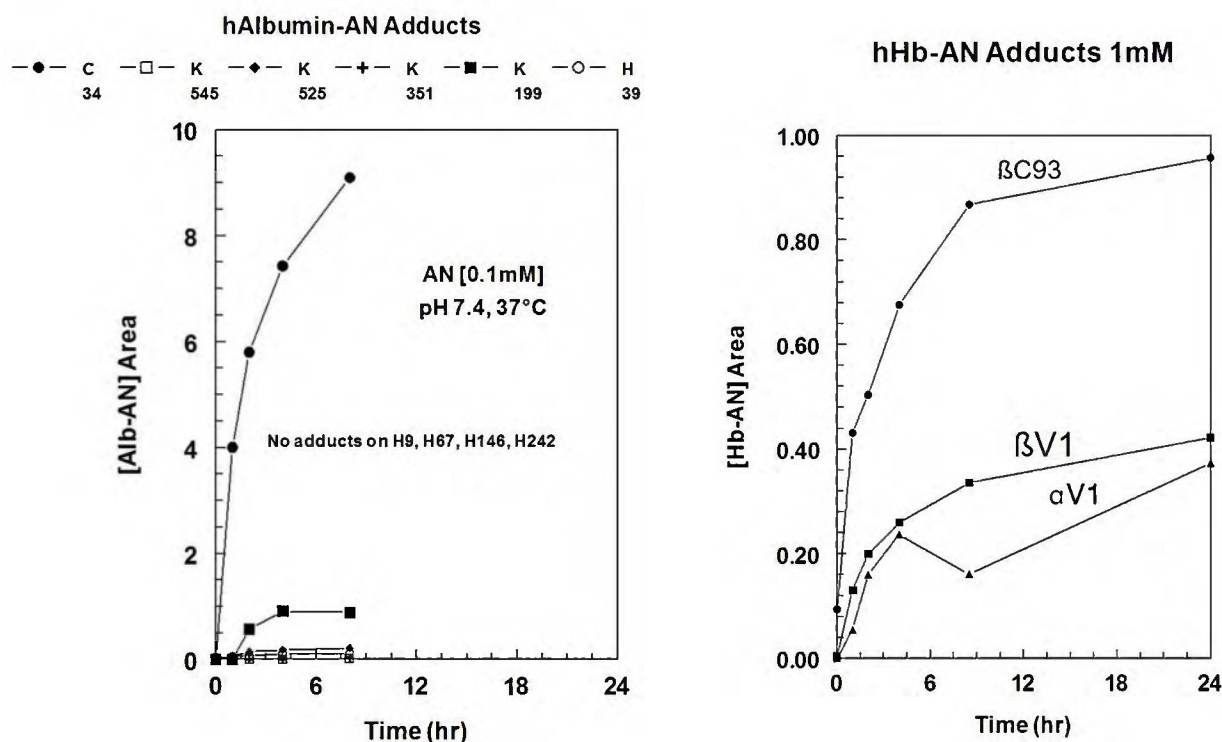


The site of the AN-adduct on this peptide is again readily apparent from the 4Da shift, which begins at the amino acid site of adduction. Specifically, the y₂+1 ion in both T10 and T10* is at m/z = 262.14, whereas the y₃+1 ion in T10* (m/z = 418.17) is shifted 4Da from the same ion in T10 (m/z = 422.17), indicating that C93 is the site of the AN covalent adduct. This is confirmed by the b-ion series, where b₁₀+1 ion occurs at m/z = 1057.53 in both T10 and T10*, whereas the b₁₁+1 ion in T10* (m/z = 1213.57) is shifted 4Da from the equivalent ion in T10 (m/z = 1217.59).

From this analysis, we now had a method in hand to monitor AN exposure by assessing the level of AN adduction of β C93 in human hemoglobin.

To verify that HSA-C34 and Hb β -C93 are indeed the most reactive sites for reaction with AN in human serum albumin and human hemoglobin, respectively, we conducted the following experiment.

Whole blood was incubated with 0.1 or 1 mM acrylonitrile for up to 24h. Samples were taken periodically and plasma was separated from red blood cells by centrifugation. Plasma proteins were precipitated with acetonitrile and RBC proteins were precipitated with acid-acetone. Precipitated proteins were treated as previously described for tryptic digestion and LC/MS analysis. However in this experiment, we monitored the reaction of AN with all detectable amino acid sites in both albumin and hemoglobin. The results of those experiments are illustrated below.



The figure on the left, we observed that AN could form adducts at not only Cys34 but also at Lys199, 351, 525, 545 and His39. No adducts with His9, 67, 146 or 242 were observed. It is interesting to note that these residues are the most reactive sites for the electrophile 4-hydroxy-2-nonenal (HNE). However, the data clearly indicate that Cys34 is the most reactive site in human albumin for AN.

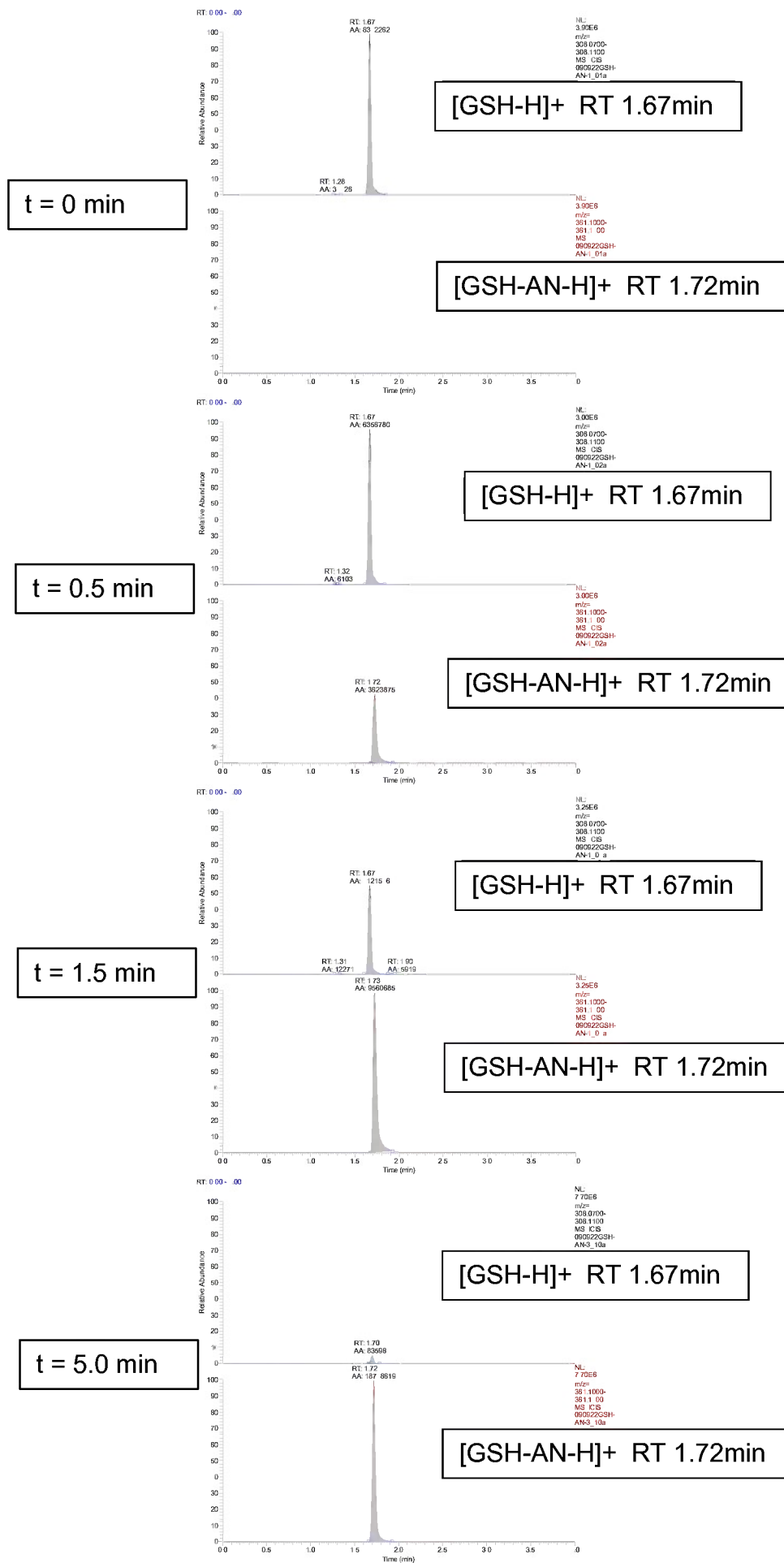
In a similar way, the figure on the right indicates that we could observe AN adducts not only on β Cys93 but also on the N-terminal Valines on both the alpha (α) and beta (β) chains of hemoglobin as expected. Again, β Cys93 was the most reactive site in hemoglobin. At this concentration of AN, no adducts at Lysines 8, 59, or 144 on the beta chain, nor Histidine 20 on the alpha chain were detected, although small amounts of these adducts could be detected following incubation with 10mM AN over the same time period.

Second Order Rate Constants for the In Vitro Reaction of the Toxic Industrial Chemical Acrylonitrile with the Most Reactive Sites in Human Blood:

Having now devised methods for the measurement of AN-adducts of blood GSH, HSA-C34 and Hb β -C93 and having verified that these three cysteines are the most reactive nucleophiles in blood for the TIC acrylonitrile, we needed to put the relative reactivity of AN with these blood constituents on a quantitative basis. To do this we measured the second order rate constants for the in vitro reaction of AN with human blood hemoglobin, albumin and GSH.

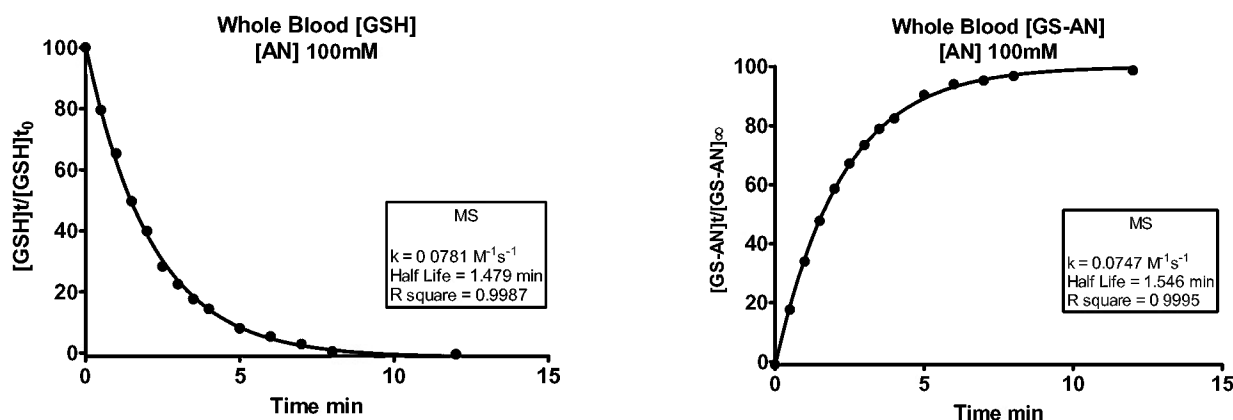
Rate Constants for the Reaction of AN with GSH in Human Whole Blood:

Fresh human blood was incubated with a high concentration of AN (100mM), pH 7.4, 37°C in order to satisfy pseudo first-order conditions. The kinetics of the formation of the AN-adduct with blood GSH was monitored as follows. Ion Chromatograms of TCA supernatants of a reaction of Whole Blood at 37°C at pH 7.4-7.5 with 100mM AN were analyzed at various times during the reaction. A typical set of ion chromatograms are illustrated on the next page.



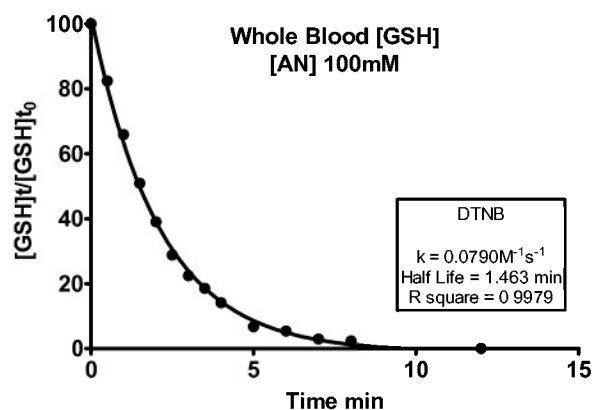
Note as the reaction progresses, the area of the GSH peak decreases, whereas the area of the GSH-AN adduct increases. The area ratio (GSH or GSH-AN over their respective internal standards) as a function of time were used to calculate the second order rate constants for the disappearance of GSH and the appearance of the GSH-AN adduct.

Typical plots of the area ratio as a function of time for the disappearance of GSH and the appearance of the GSH-AN adduct determined by Mass Spectrometry (MS) are illustrated below.



The second order rate constant for the disappearance of GSH in this individual experiment was $0.0781 \text{ M}^{-1}\text{s}^{-1}$, in good agreement with the second order rate constant for the appearance of the GSH-AN adduct of $0.0747 \text{ M}^{-1}\text{s}^{-1}$.

In a similar way, the rate constant for the disappearance GSH was measured spectrophotometrically using the colorimetric reagent DTNB, which forms a yellow colored product upon reaction with GSH. A typical plot of the disappearance of GSH as a function of time of the reaction of 100mM AN with whole blood is shown below.



The second order rate constant measured for this individual experiment was $0.0790 \text{ M}^{-1}\text{s}^{-1}$, in excellent agreement with the rate constants measured by mass spectrometry.

We are still in the process of repeating these experiments in order to obtain an estimate of the variability in measuring these rate constants. The average second order rate constants for each of these measurements for several of the experiments that we have conducted to date is summarized in the following table.

Second Order Rate Constants ($M^{-1}s^{-1}$)		
\downarrow [GSH]	\downarrow [GSH]	\uparrow [GSH]
Spectrophotometric	Mass Spectrometry	Mass Spectrometry
0.0813	0.0799	0.0776

Note again the excellent agreement between the two techniques for measuring the disappearance of GSH and the agreement of both with the rate of appearance of the GSH-AN adduct. We thus are getting a good handle on the rate of reaction of AN with whole blood GSH, one of the three most reactive sites.

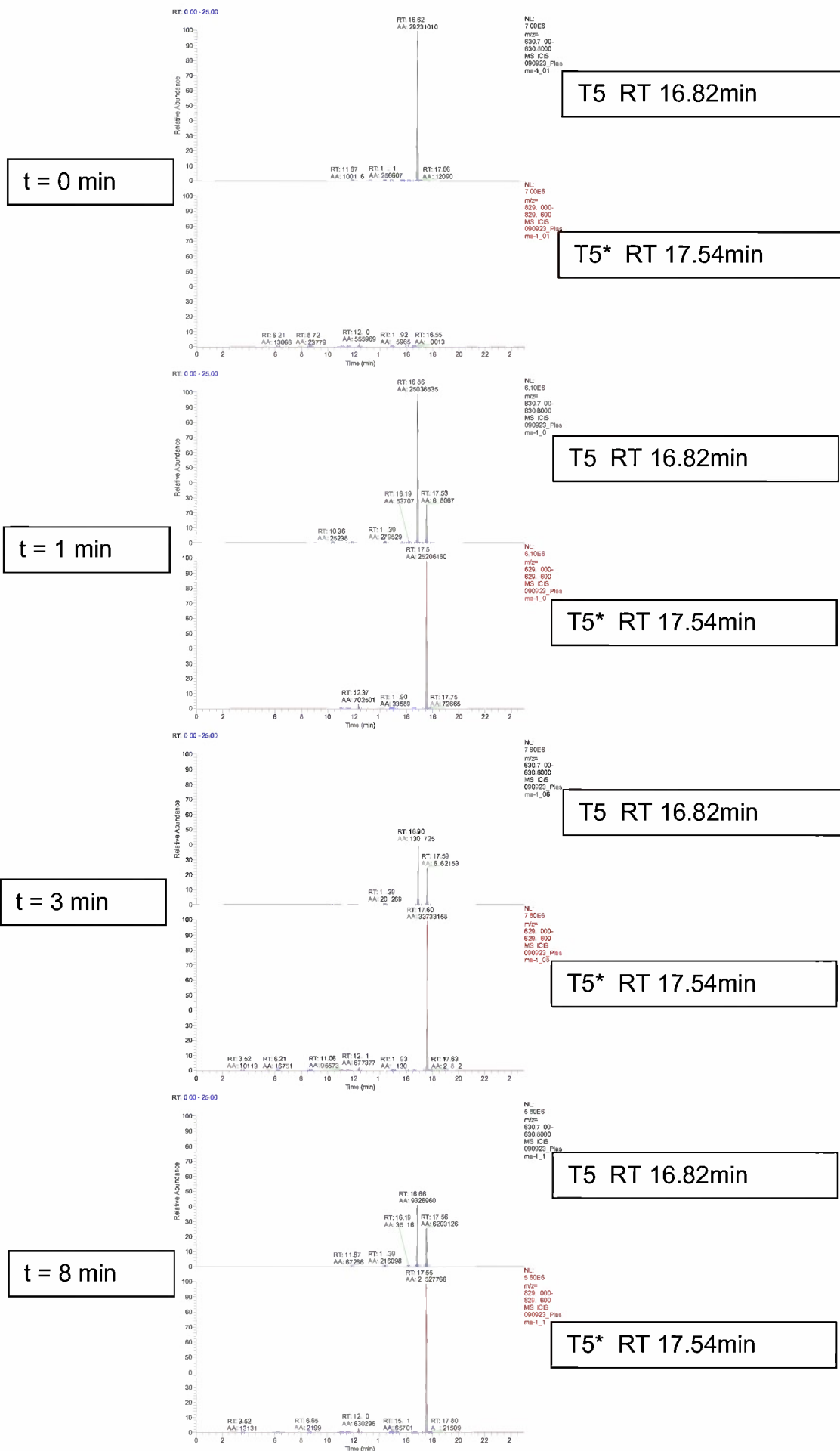
Rates of Reaction of AN with Human Serum Albumin Cysteine-34 (HSA-C34) and Human Hemoglobin Beta Chain Cysteine-93 (Hb β -C93).

We have experienced some technical difficulties in measuring the second order rate constants for the reaction of AN with these two protein sites using whole blood as was done for GSH. We are currently conducting experiments to eliminate these technical problems and are confident that we will be successful.

We have, however, been successful in measuring the rate constant for the reaction of AN with Human Serum Albumin Cysteine-34 (HSA-C34) in human plasma and the rate of reaction of AN with Human Hemoglobin Beta Chain Cysteine-93 (Hb β -C93) in RBC lysates. We have no reason to anticipate that the rate constants measured in plasma and RBC lysates will differ significantly from the rates to be measured in whole blood, but we will eventually measure rate constants in whole blood as well in order to be certain.

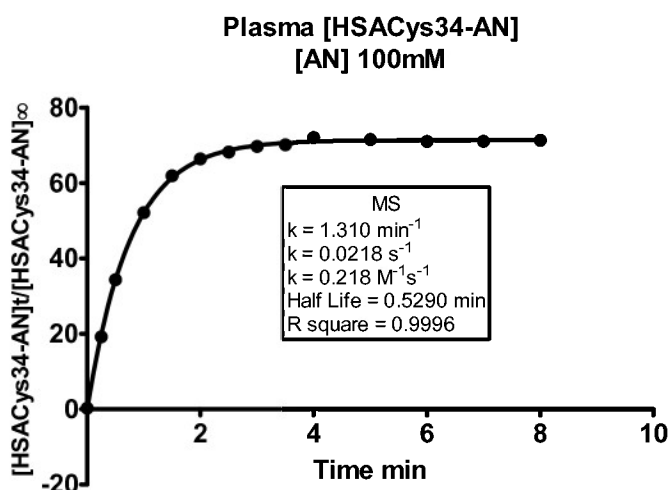
Rate Constant for the Reaction of AN with HSA-C34 in Human Plasma:

The rate of formation of the AN-adduct of HSA-C34 was measured in human plasma. Fresh human plasma was incubated with 100mM AN at 37°C and pH 7.4-7.5. This concentration of AN was chosen so that the reaction would follow pseudo first-order kinetics. Samples were taken at various times after starting the reaction and plunged into acetonitrile to stop the reaction and precipitate the plasma proteins. The plasma protein precipitate was subsequently reduced with dithiothreitol and the free cysteines were alkylated with iodoacetamide. The reduced and alkylated plasma proteins were then digested with trypsin and an aliquot of the tryptic digest was applied to a Hypersil Gold C18 reverse-phase column (50 x 2.1mm) and the peptides were eluted with an acetonitrile gradient containing 0.1% formic acid. Selective Ion Monitoring Chromatograms of a plasma protein tryptic digest of a reaction of plasma at 37°C at pH 7.4-7.5 with 100mM AN at various times during the reaction is shown below.



Note as the reaction progresses, the area of the monoisotopic T5 peak ($m/z = 830.8$) decreases, whereas the area of the monoisotopic T5* ($m/z = 829.4$) AN-adduct increases. At one minute into the reaction of AN with plasma proteins, about 50% of HSA-C34 is modified by AN. The small peak at 17.53 min in m/z 830.8 ion chromatogram is from an isotope peak of T5*. By three minutes into the reaction, AN has reacted with essentially all of the free HSA-C34 in the plasma sample as evidenced by the similarity of the chromatogram at 3 minutes versus the chromatogram at 8 minutes. The reason why the T5 peak does not completely disappear at the end of the reaction with AN is due to the fact that approximately 15-30% of HSA-C34 in human plasma is combined with GSH in a mixed disulfide bond. Those cysteine residues will be protected from reaction with AN but will be liberated by reduction of the plasma proteins with DTT prior to alkylation with iodoacetamide.

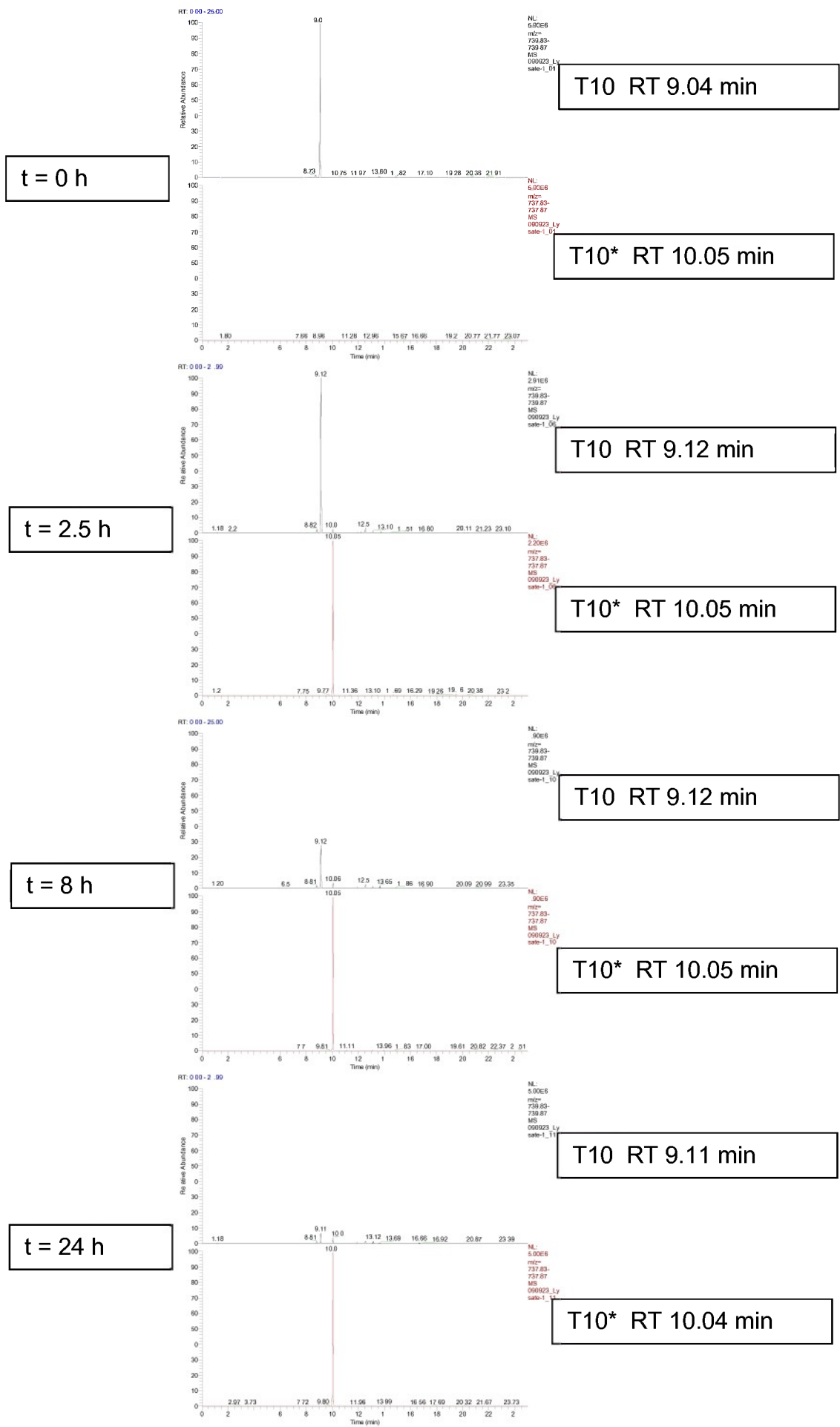
These area changes as a function of time were used to calculate the second order rate constants for the appearance of the HSA-C34-AN adduct. Typical plots of the area changes as a function of time for the appearance of the HSA-C34-AN adduct determined by mass spectrometry are illustrated below.



The second order rate constant for the appearance of HSA-C34-AN adduct in this individual experiment was $0.218 \text{ M}^{-1}\text{s}^{-1}$. The average rate constant for this reaction for all of the experiments we have conducted to date will be summarized after the following description of the reaction of AN with human hemoglobin in RBC lysates.

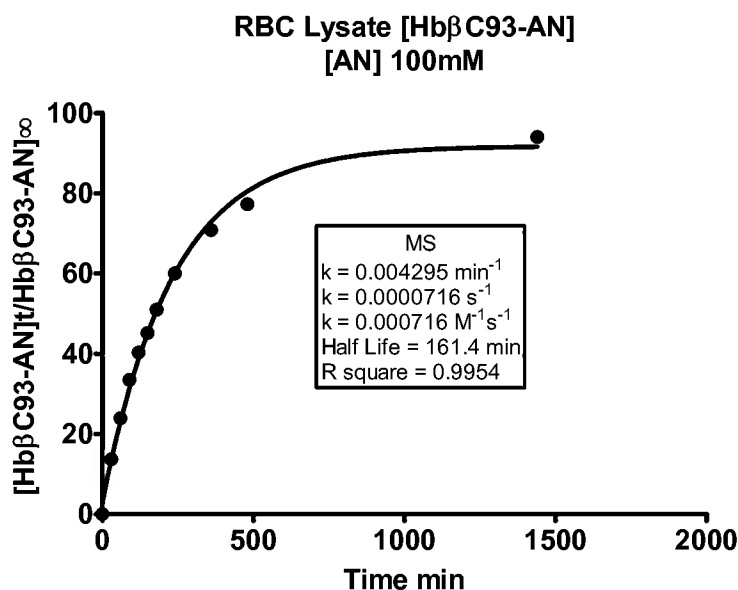
Rate Constant for the Reaction of AN with Hb β -C93 in Human Red Blood Cell Lysates:

The rate of formation of the AN-adduct of Hb β -C93 was measured in human RBC lysates. Fresh human RBC lysate was incubated with 100mM AN at 37°C and pH 7.4-7.5. This concentration of AN was chosen so that the reaction would follow pseudo first-order kinetics. Samples were taken at various times after starting the reaction and plunged into acidified acetone to stop the reaction and precipitate the red blood cell proteins. The RBC protein precipitate was then alkylated with iodoacetamide. The alkylated rbc proteins were then digested with trypsin and an aliquot of the tryptic digest was applied to a Hypersil Gold C18 reverse-phase column (50 x 2.1mm) and the peptides were eluted with an acetonitrile gradient containing formic acid. Selective Ion Monitoring Chromatograms of an RBC lysate protein digest at various times during the reaction is shown below.



Note as the reaction progresses, the area of the monoisotopic T10 peak ($m/z = 739.85$) decreases, whereas the area of the monoisotopic T10* ($m/z = 737.85$) AN-adduct increases. At 2.5h into the reaction of AN with RBC lysate proteins, about 50% of Hb β -C93 is modified by AN. In contrast the extremely rapid reaction of AN with HSA-C34, the reaction of AN with Hb β -C93 requires over 24h to reach a plateau.

These area changes as a function of time were used to calculate the second order rate constants for the appearance of the Hb β -C93-AN adduct. A typical plot of the area changes as a function of time for the appearance of the Hb β -C93-AN adduct determined by mass spectrometry is illustrated below.



The reaction of AN with human plasma and RBC lysate has been repeated several times and the average second order rate constants for each of these measurements is summarized in the following table.

Second Order Rate Constants ($M^{-1}s^{-1}$)	
\uparrow HSA-C34-AN	\uparrow Hb β -C93-AN
Mass Spectrometry	Mass Spectrometry
0.224	0.000722

Summary of Rate Constant Measurements to date:

In summary, the second order rate constants in $M^{-1}s^{-1}$ were as follows: disappearance of GSH in whole blood, 0.0813 (spec) and 0.0799 (MS); appearance of GS-AN in whole blood, 0.0776 (MS), appearance of Hb β -C93-AN in RBC lysate, 0.000722 (MS) and appearance of HSA-C34-AN in plasma, 0.224 (MS). The data indicate that the most reactive site for AN in human blood is C-34 of plasma albumin. This site reacts 2.8 times faster than GSH and 310 times faster than Hb β -C93.

Future studies will be directed toward repeating these measurements to get a better estimate of the accuracy and precision of these rate constants. We also will continue our attempts to measure the rate constants for HSA-Cys34 and Hb β -C93 in whole blood rather than plasma and RBC lysates, respectively.

Experiments Directed Toward Minimizing the Analysis Time for the Biomarker of AN Exposure:

We have previously identified human serum albumin cysteine-34 (HSA Cys34) as the most reactive site in whole blood for covalent interaction with certain toxic industrial chemicals (TICs), specifically acrylonitrile. Since an important aim of this project is to develop a biomarker for exposure to such TICs, we conclude that HSA-Cys34 will serve this purpose. However, if this biomarker is to be useful to triage individuals who have been exposed to such TICs, it will be important to be able to measure this biomarker rapidly.

Toward this end, we have conducted experiments to speed up the measurement of this biomarker. One question to be answered was whether it would be necessary to purify the albumin from plasma prior to analysis. Second, was how to speed up the next two slowest steps in the process, namely the time to digest the proteins with trypsin and the liquid chromatographic step to separate the TIC-adducted peptides, specifically the peptide containing HSA-Cys34.

Albumin Purification:

Human serum albumin was purified from plasma as follows. Briefly, fresh human plasma was diluted 1:1 v/v with phosphate-buffered saline (PBS), pH 7.4. An equal volume of saturated ammonium sulfate, pH 7.0, was added dropwise to precipitate the globulins. The supernatant was separated from the precipitated globulins by centrifugation. Sufficient solid ammonium sulfate was added to the supernatant to bring the concentration to 80%. The pH of the 80% fraction was adjusted to pH 4.7 to precipitate albumin and other minor contaminants. The albumin precipitate was separated by centrifugation. The albumin was then dissolved in 0.1M sodium phosphate, pH 7.4 and then applied to a Affi-Gel Blue column (high affinity for albumin), equilibrated with the same buffer. The column was then washed with the same buffer to elute proteins that are not retained by Affi-Gel Blue. The albumin was then eluted from the column with 2M NaCl in 0.1M sodium phosphate, pH 7.4. The albumin fractions were then concentrated in an Amicon stirred cell and desalted on a Sephadex G-25 column, eluted with ammonium acetate, pH 6.9. The purified albumin (HSA-1) was then subjected to the same treatments as total plasma proteins (HSA-2), precipitated from whole plasma with acetonitrile (ACN) as described below.

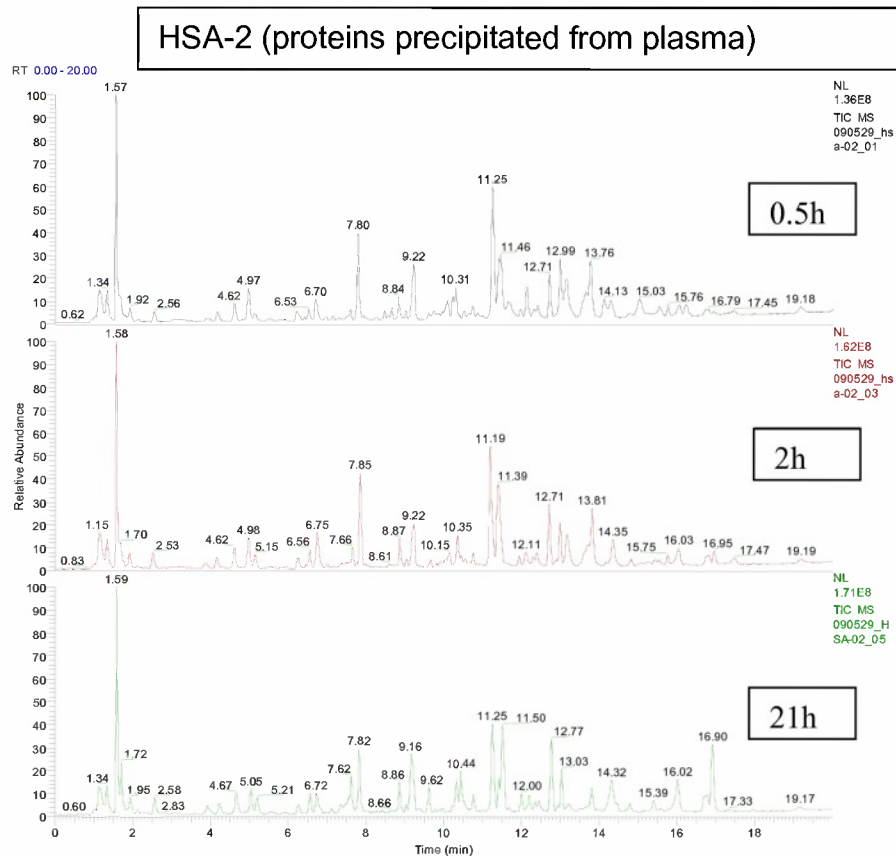
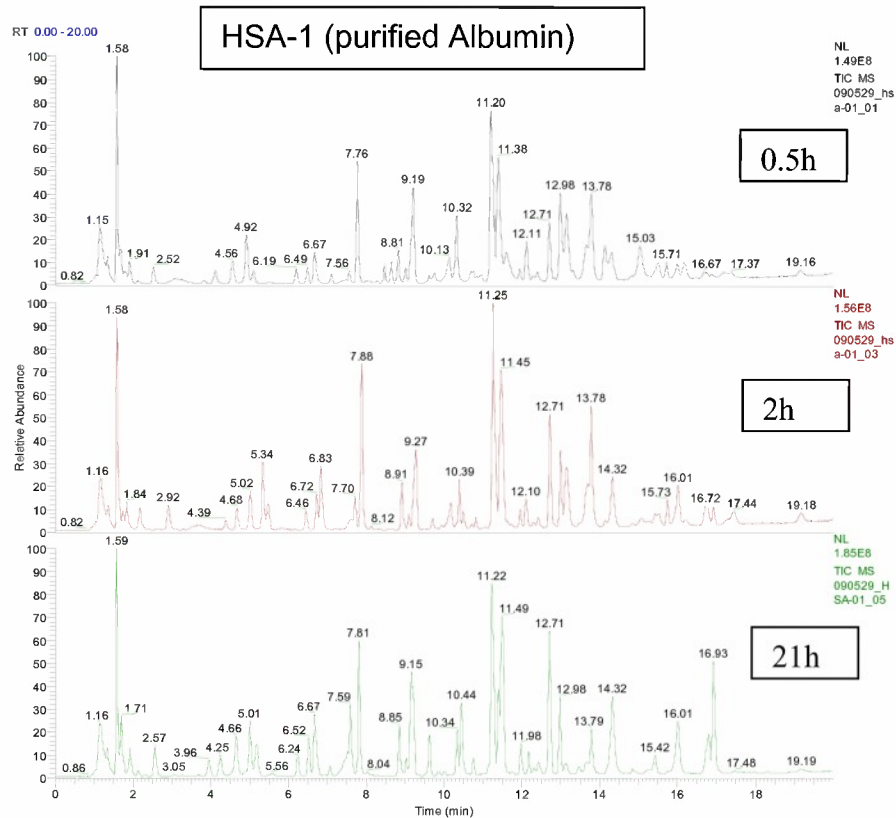
Trypsin Digestion:

Typically digestion of plasma or tissue proteins with trypsin is conducted over several hours or in some cases overnight. This time scale would not be suitable for rapid triage of exposed individuals. We have thus conducted experiments to determine the minimum time required to completely liberate the HSA tryptic peptide T5, containing Cys34, from the rest of the protein.

A literature survey indicated that adding acetonitrile (ACN) to the tryptic digestion mixture would increase the rate of trypsin digestion. Thus experiments were conducted comparing

digestion rates of purified albumin (HSA-1) vs. precipitated plasma proteins (HSA-2) in 25% acetonitrile. Specifically, samples of approximately 50 ug protein were dissolved in 250 uL 50 mM NH_4HCO_3 , mixed with 2.5 uL 100 mM dithiothreitol (DTT, to reduce the disulfide bridges, final concentration 1 mM), incubated at 70°C for 15 min, mixed with 7.5 uL 100 mM iodoacetamide (IAA, to block the free cysteines, final concentration 3 mM), incubated at RT for 10 min, mixed with 105 uL 50 mM NH_4HCO_3 , 125 uL ACN (final ACN concentration 25% v/v), and 10 uL trypsin (about 5 ug, protein:trypsin = 10:1), and incubated at 37°C. Samples of 100 uL were collected at 0.5, 1, 2, 3.5, and 21 hours, diluted with 400 uL 0.1% formic acid, and analyzed by LC/MS and LC/MS/MS.

Typical total ion-current chromatograms of the two samples are shown on the next page.

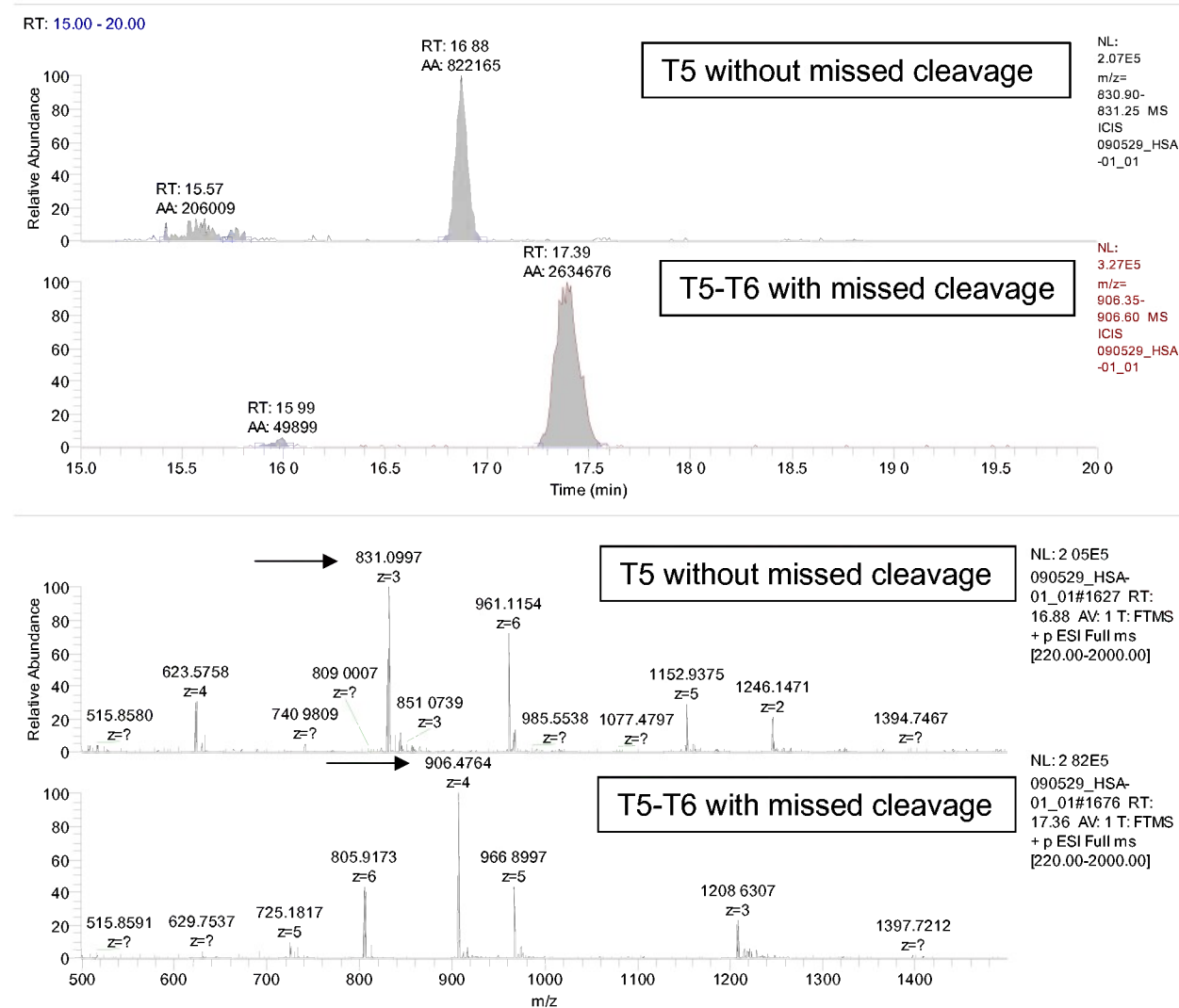


Total Ion Chromatograms of HSA-1 and HAS-2 as a function of tryptic digestion time.

Little protein other than HSA was detected in HSA-1, while several other proteins known to exist in plasma were detected in HSA-2. The major peaks were very similar but there were some differences after prolonged incubation. MS/MS spectra of the 0.5 hour samples from data dependent scans matched over 90% of HSA sequence. After prolonged incubation, sequence coverage decreased slightly along with less identified peptides with missed cleavages. These results indicated that there are large differences in the accessibility of trypsin to different regions of HSA. Parts of the HSA can be digested very easily whereas other regions require more time to be digested completely.

HSA-1 was digested with 25% ACN added at pH 8.0. Two peptides containing Cys34 were identified by MS/MS, one from complete digestion (T5) and one with one missed cleavage (T5-T6).

Total Ion Chromatograms of HSA-1 digest and MS/MS Spectra of Peaks at RT 16.88 (T5) and RT 17.39 (T5-T6)



Top: Total Ion Chromatograms of HSA-1 digest and Bottom: MS/MS Spectra of Peaks at RT 16.88 (T5) and RT 17.39 (T5-T6).

Summary of the results of the time dependence of the digestion HSA-1 and HSA-2 by trypsin in the presence of 25% ACN at pH 8.0.

Peak area changes at different time points in the digestion are shown in the table below.

Time (h)	Area (m/z 831.1)	Area (m/z 906.5)	Area Ratio
	Z = 3	Z = 4	906/831
0.5	822165	2634676	3.205
1	2793602	5616857	2.011
2	8863135	8715644	0.983
3.5	20129847	12603019	0.626
21	67960984	1343815	0.020

Time (h)	Area (m/z 831.1)	Area (m/z 906.5)	Area Ratio
	Z = 3	Z = 4	906/831
0.5	969802	2259055	2.329
1	3316504	4775455	1.440
2	6196579	4341499	0.701
3.5	15778311	7750850	0.491
21	36946210	341543	0.009

HSA-1

HSA-2

The progress of the digestion can be determined by the Area Ratio of the peak at RT 17.39 min (T5-T6, m/z 906.5) divided the peak at RT 16.88 min (T5, m/z 831.1). This ratio will approach zero as the digestion reaches completion. It was noted that the total area of the sum of the peaks at RT 16.88 and 17.39 increased with digestion time. This indicates the continuing formation of these peptides. The trivial amount of T5-T6 detected after 21 hours indicates complete digestion. Unfortunately, HSA-Cys34 happens to be located in a region that is more difficult for trypsin digestion.

It should be pointed out that the data at 0.5 hour could be claimed as a complete success of the digestion method for the purpose of protein identification. That is the criteria used by most vendors and published papers. However, for our purposes we require complete digestion of HSA in order to utilize the T5-adduct at Cys34 as a quantitative biomarker of the level of exposure to a TIC.

Since the data above suggested that Cys34 was in a region of the protein that resisted digestion, we attempted to speed up the digestion by adding 2M urea to partially unfold the HSA to allow better access to trypsin. The results of those experiments are summarized in the Table below.

Time (h)	Area (m/z 831.1)	Area (m/z 906.5)	Area Ratio
	Z = 3	Z = 4	906/831
0.5	84196	3178385	37.750
1	236881	4583274	19.348
2	489764	6768569	13.820
3.5	481199	6011959	12.494
21	5709888	18555732	3.250
Time (h)	Area (m/z 831.1)	Area (m/z 906.5)	Area Ratio
	Z = 3	Z = 4	906/831
0.5	83008	2706817	32.609
1	129712	2958729	22.810
2	287061	4099315	14.280
3.5	1096091	12469659	11.376
21	1690539	10805892	6.392

HSA-1

HSA-2

The results indicate that digestion was worse in the presence of 2M urea. The most likely explanation for this result is that the trypsin itself was being denatured by the urea and thus had decreased proteolytic activity.

After considerable trial and error it was finally discovered that HSA could be digested quite rapidly at pH 8.5 without additional ACN or urea. A summary of the results obtained under these conditions is shown in the Table below.

Time (h)	Area (m/z 831.1)	Area (m/z 906.5)	Area Ratio
	Z = 3	Z = 4	906/831
1	24578002	20903	0.001
3	17596210	12896	0.001
5	14894255	0	0.000
7	18038897	0	0.000
23	8509245	12436	0.001
Time (h)	Area (m/z 831.1)	Area (m/z 906.5)	Area Ratio
	Z = 3	Z = 4	906/831
1	12551478	3300	0.000
3	8562109	0	0.000
5	6632058	0	0.000
7	6942218	0	0.000
23	4386722	17262	0.004

Control Plasma

AN Treated Plasma
1mM/24h

Since the digestion was already completed at the 1h time point, an additional experiment was conducted at shorter times and the results are tabulated below.

Time	Area (m/z 831.1)	Area (m/z 906.5)	Area Ratio
	Z = 3	Z = 4	906/831
0.25	169415755	355301	0.002
0.5	154257347	60293	0.000
1	136325375	103554	0.001
2	134103116	88662	0.001
4	110572381	65437	0.001

Control Plasma

It can be seen that the digestion was complete in 15 minutes or less.

In summary, we have found conditions, whereby HSA can be completely digested within 15 minutes and thus eliminates one of the possible bottlenecks in sample processing. In addition, the rate of digestion of HSA in whole plasma (HSA-2) was the same as purified HSA (HSA-1), so as far as the digestion step is concerned, plasma is a suitable sample and time consuming albumin purification is not necessary.

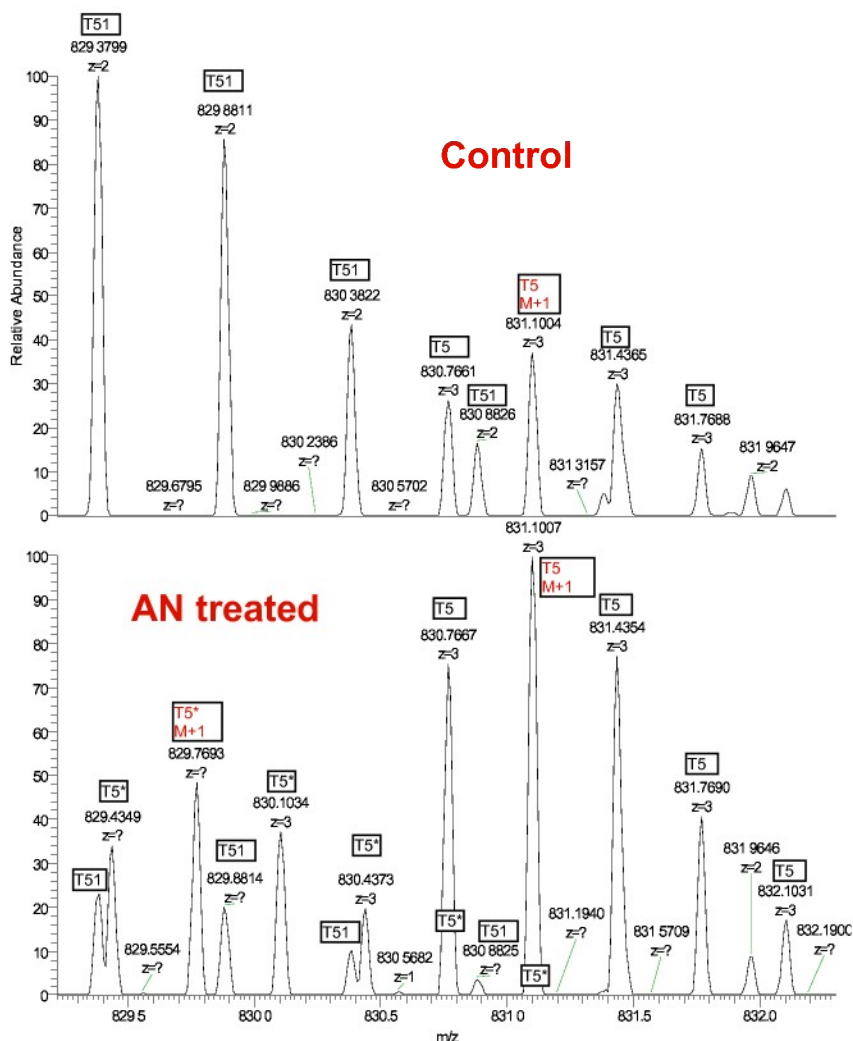
Reversed-Phase Liquid Chromatography:

The remaining possible bottleneck is the time consuming process of liquid chromatography. Until now, the biomarker peptide T5 had always been separated from other peptides (e.g. T5-T6) in the digest by reversed-phase liquid chromatography (RPLC), (see earlier figures). In addition, for TIC exposure assessment, the TIC-adducted peptide (T5*) would have to be separated from the non-TIC-adducted peptide (T5). Recall, T5 represents HSA peptide T5, which has its Cys34 blocked with iodoacetamide, whereas T5* represents HSA peptide T5, which has reacted with the TIC, in this case acrylonitrile (AN).

A complete RPLC run of a peptide digest would take about 45 minutes. The following experiments were conducted to see if the RPLC step could be eliminated by using the high resolution capabilities of the Orbitrap Mass Spectrometer as the separation as well as quantification device.

Toward this end, AN was reacted with whole blood at several concentrations and the reaction stopped at various times by rapidly separating plasma from red blood cells in a microcentrifuge. The plasma proteins were then rapidly precipitated with acetonitrile. The plasma protein precipitates were then reduced with DTT, alkylated with iodoacetamide and digested with trypsin as described earlier.

The tryptic digest was then introduced into the Orbitrap by direct infusion (no RPLC column). A portion of an MS spectrum of such a sample is shown below.



MS spectra of a control plasma protein digest and a plasma protein digest derived from AN-treated whole blood. Note the ion clusters of T51 ($z=2$, m/z 829.3799, 829.8811, 830.3822, and 830.8825), unadducted T5, $z=3$, m/z 830.7661, **831.1004**, 831.4365, 831.7688, and 832.1031), and T5*, adducted with AN ($z=3$, m/z 829.4339, **829.7693**, 830.1034, 830.4373, and the M+4 isotope peak of T5*, which is under the monoisotope peak of T5).

Although there is considerable overlap of the ion clusters in this complex digest, quantification of the relative amount of the AN-adduct can still be done by comparing the intensity of the M+1 isotope peaks of T5* (m/z **829.7693**) vs. the corresponding T5 (m/z **831.1004**).

It is important to note the following:

- M+1 is the most abundant peak for both T5 and T5*
- the monoisotope peak of T5* (m/z 829.4339) overlaps the monoisotope peak of T51 (m/z 829.3779)
- the M+4 peak of T5* overlaps the monoisotope peak of T5
- the M+5 peak of T5* overlaps the M+1 peak of T5, but the abundance of M+5 is much less than that of M+4 thus less error is caused by the overlap of the T5* isotope peak with T5 peak used for quantification.

It is also important to note, that the analysis of this complex sample would not be possible were it not for the high resolution provided by the Orbitrap instrument purchased with funds provided by this award.

Recall, as previously describe on page 9, the expected mass shift for a Cys residue that has reacted with AN vs. that blocked by IAM is 4Da. However, this peptide T5 carries a triple charge, thus the 4Da must be divided by 3 to the get expected m/z difference of 1.33 as observed in the spectra above.

T5 M+1	831.1004					
T5* M+1	829.7693					
Δ	1.3311	X	Z=3	3.9938	4 Da shift	

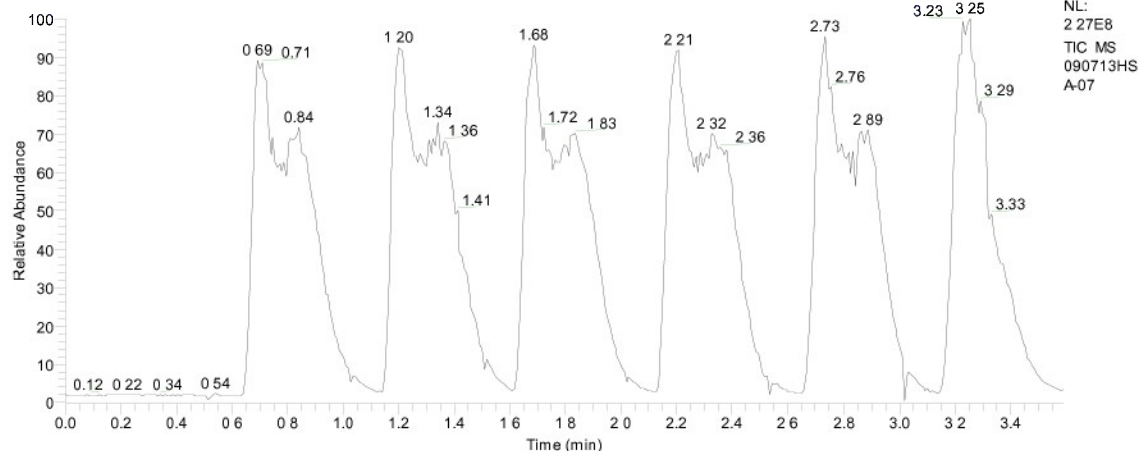
Comparison of rapid infusion techniques:

Since the experiments above indicated that the RPLC step could be eliminated by simply infusing the sample into the Orbitrap, three separate rapid infusion techniques were evaluated and are briefly described below. In all of the following three methods, the RPLC column was eliminated from the system.

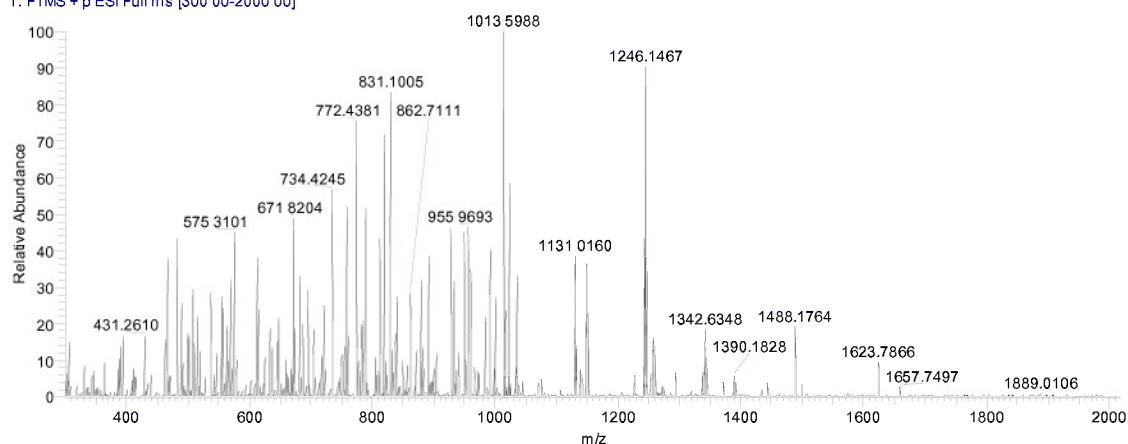
Loop injection:

A 5 uL sample loop was used. The sample loop was flushed and filled with the sample and carried into the ion source by a flow of 50% ACN/0.1% Formic Acid at 50 uL/min with an Accela pump. Several injections could be made within a couple of minutes (see next page). Samples can be analyzed quickly with this technique but it requires manual injection.

RT: 0.00 - 3.59



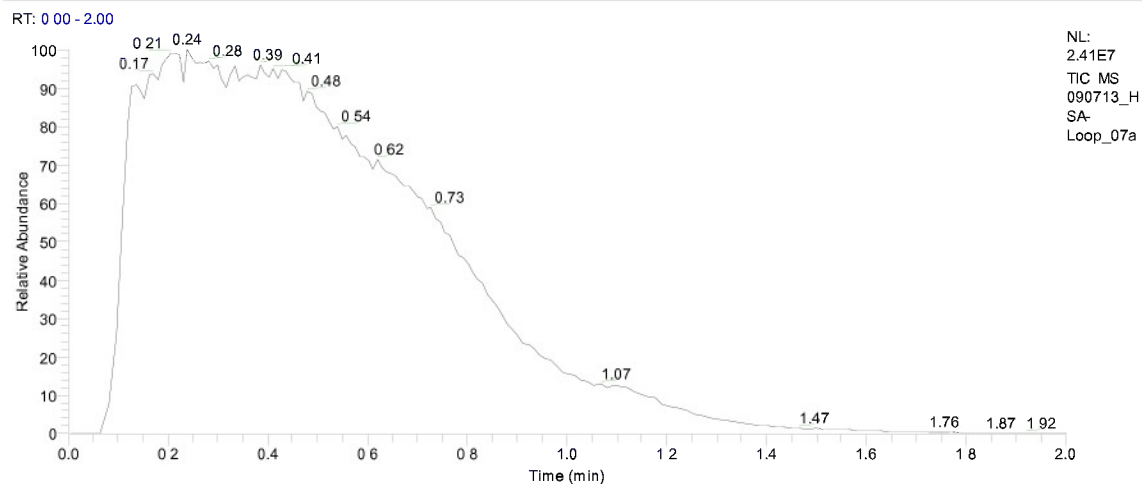
090713HSA-07 #76-99 RT: 0.68-0.87 AV: 24 NL: 2.02E6
T: FTMS + p ESI Full ms [300.00-2000.00]



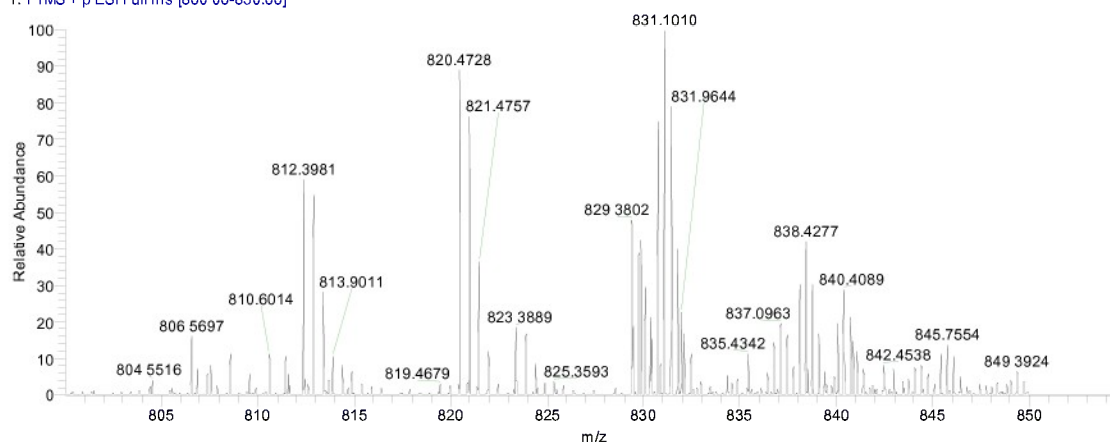
Top figure: Total Ion Current observed with multiple injections of a plasma protein digest.
Bottom figure: Full MS spectrum following one of the injections. The data indicate that with this technique, individual samples could be injected/analyzed approximately every 45-60 seconds.

LC injection without column (automated sample injection):

The LC autosampler was used to replace the sample loading valve in the loop injection method. Basically, the LC/autosampler system was used to automate the injection of individual samples. With this technique, it takes about 3 min to analyze a sample (1 min for sample loading and 2 min for infusion into the ion source (see next page). With this technique, sample loading and data acquisition are fully automated.

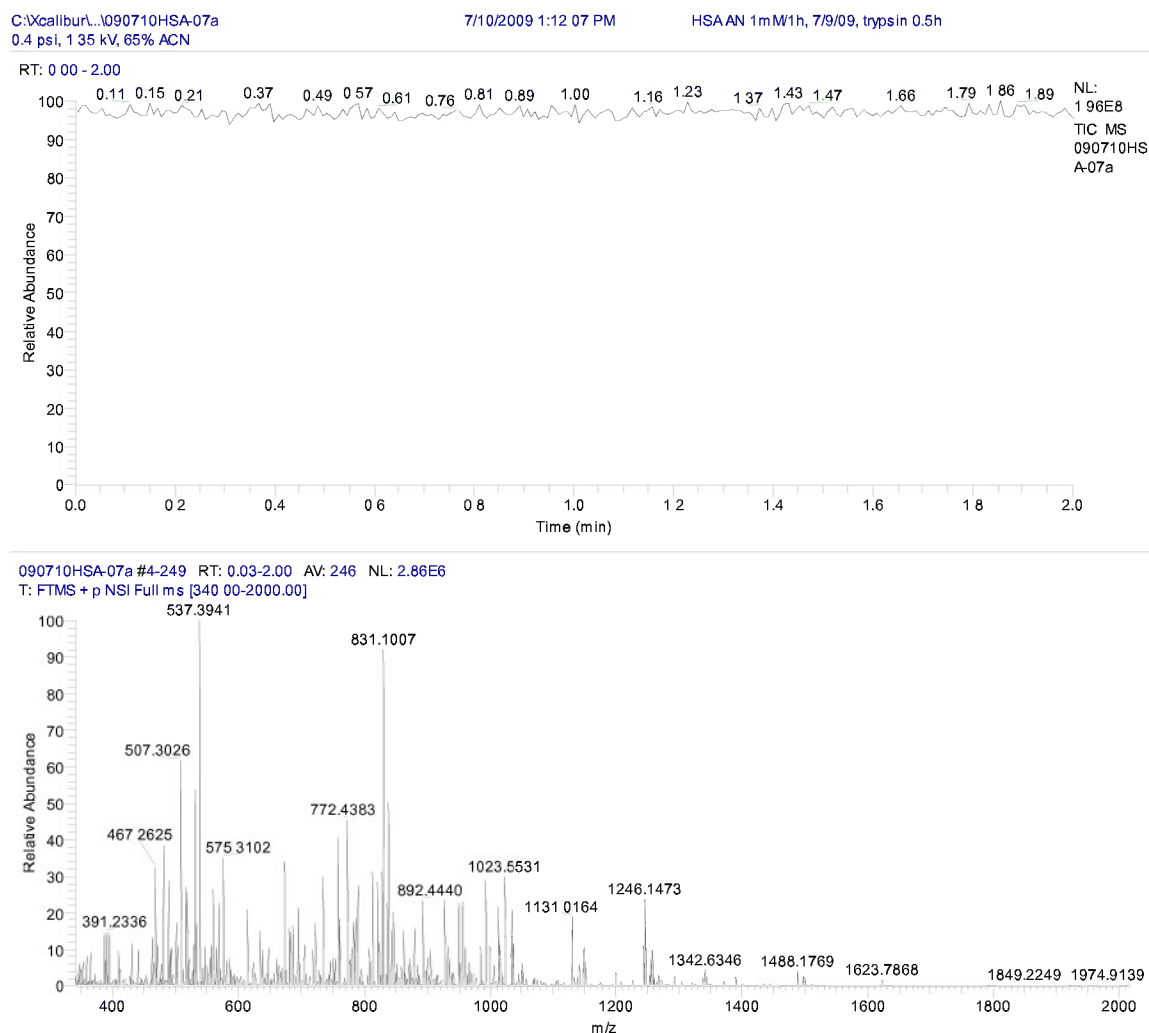


090713_HSA-Loop_07a #10-59 RT: 0.13-0.55 AV: 50 NL: 1.24E6
T: FTMS + p ESI Full ms [800.00-850.00]



Top Figure: Total Ion Current of a single sample digest delivered via the fully automated LC/autosampler technique. The relevant peaks for adduct quantitation are readily observed at m/z 829.7693 (T5*, not annotated in figure) and m/z 831.1010 (T5).

Lastly, the TriVersa Nanomate was used to analyze the samples. The results of a typical experiment are illustrated below.



Top Figure: Total Ion Current of a sample delivered to the ion source via the TriVersa Nanomate. Bottom figure: Complete MS spectrum of the injected sample

When the spray is stable, quite reproducible results can be obtained. However, sometimes, it is difficult to obtain a stable spray from the Nanomate and thus it is not as reliable as the LC/autosampler injection technique. However, this is also a fully automated method and is capable of acquiring data in a couple of minutes per sample.

To fully compare the techniques, a group of samples resulting from the incubation of whole blood with AN at several concentrations and incubation times were analyzed with all three techniques. The results are shown in the Tables below.

Comparison of results from loop injection, LC/autosampler and LC/MS analysis (gold standard).

Sample	loop injection			LC/Autosampler-1)			LC/Autosampler-(2)			LC/MS
	N	Ave (%)	SD	N	Ave (%)	SD	N	Ave (%)	SD	
Control	6	0.01	0.03	2	0.00	0.00	2	0.00	0.00	0.020
AN 0.1mM/1h	6	2.18	0.81	2	2.28	0.24	2	3.61	0.09	4.004
AN 0.1mM/2h	6	4.51	0.69	2	4.50	0.01	2	5.23	0.00	5.801
AN 0.1mM/4h	6	5.92	0.52	2	6.32	0.11	2	6.82	0.02	7.430
AN 0.1mM/8h	6	7.63	0.60	2	7.58	0.23	2	8.20	0.14	9.095
AN 0.1mM/8.5h	6	7.75	0.51	2	7.72	0.34	2	8.00	0.10	8.578
AN 1mM/1h	6	29.77	1.62	2	29.78	0.03	2	28.90	0.51	28.332
AN 1mM/2h	6	39.27	1.02	2	39.14	0.29	2	39.50	0.03	38.788
AN 1mM/4h	6	48.23	0.82	2	48.00	0.52	2	47.62	0.03	45.783
AN 1mM/8.5h	6	53.72	0.94	2	53.44	0.29	2	52.98	0.27	50.478
AN 1mM/24h	6	57.48	0.84	2	56.98	0.43	2	56.76	0.16	53.681
AN 10mM/1h	6	68.44	1.96	2	69.05	0.14	2	69.09	0.70	65.642

Table Legend: Column 1 are the various treatment samples analyzed (Three concentrations of AN reacted with whole blood for various times). N is the number of replicates for each sample. Ave (%) is the % adduction of HSA-C34 by AN. SD is the Standard Deviation of the Ave (%).

Comparison of results from the Nanomate and LC/MS analysis (gold standard).

Sample	Nanomate						LC/MS
	Day 1			Day 2			
	N	Ave (%)	SD	N	Ave (%)	SD	
Control	4	0.00	0.00	3	0.00	0.00	0.020
AN 0.1mM/1h	3	3.88	0.04	4	4.05	0.11	4.004
AN 0.1mM/2h	3	10.77?	0.13	4	5.91	0.09	5.801
AN 0.1mM/4h	3	7.83	0.14	3	7.84	0.15	7.430
AN 0.1mM/8h	5	9.06	0.09	4	9.28	0.07	9.095
AN 0.1mM/8.5h	3	8.86	0.11	2	8.99	0.17	8.578
AN 1mM/1h	3	31.49	0.19	3	32.80	0.08	28.332
AN 1mM/2h	3	41.93	0.06	4	42.72	0.27	38.788
AN 1mM/4h	3	49.76	0.06	4	51.52	0.19	45.783
AN 1mM/8.5h	3	55.62	0.23	3	56.79	0.37	50.478
AN 1mM/24h	2	59.33	0.41	4	60.87	0.16	53.681
AN 10mM/1h	4	71.00	0.25	4	72.22	0.19	65.642

Table Legend: Column 1 are the various treatment samples analyzed (Three concentrations of AN reacted with whole blood for various times). N is the number of replicates for each sample. Ave (%) is the % adduction of HSA-C34 by AN. SD is the Standard Deviation of the Ave (%).

As can be seen, for the 1 and 10 mM AN treatment samples, the T5* levels from these methods are slightly higher than those determined by LC/MS. For samples from 0.1 mM AN treatment,

the T5* levels from the Nanomate are very close to those determined by LC/MS and T5* levels from loop injection are a slightly lower than those determined by LC/MS.

T5 ($z=3$, $M+1$, m/z 830.1010) and T5* ($z=3$, $M+1$, m/z 829.7693) are among the most intense peptide peaks in the spectra from direct infusion, which makes detection of low levels of T5* possible. Due to the limit of the dynamic range (a limited number of ions were used to produce a spectrum, a very small portion of the ions are from the peptides of interest, and an even smaller portion are from the peptides with the specified isotope peak and correct charge state), at present, it will be difficult to detect T5* much lower than 1% adduction. Limiting the m/z range in the data acquisition does not provide much additional sensitivity. For samples with T5* at less than 1% adduction, LC/MS is a better method with a lower limit of detection at less than 0.1% and this could be extended to even lower levels of adduction if selective reaction monitoring with a larger sample amount per injection was used. Currently, methods to selectively enrich HSA with T5* are being tested.

In summary, it is important to note that these higher levels of sensitivity would only be necessary if “trace” exposure to the TIC AN is to be assessed. From animal experiments it can be predicted that AN exposures resulting in 1% adduction of HSA-C34 would be well over one order of magnitude lower than those associated with acute toxicity. Thus the methods described above would be more than adequate to triage humans exposed to AN in a chemical attack or industrial accident.

Human Serum Albumin Cys34 Adducts as a Biomarker for Exposure to Unknown Reactive Chemicals:

In the process of conducting all of the studies described above, we also investigated the possibility that HSA-C34, located in tryptic peptide T5, might serve as a biomarker not only for acrylonitrile and in the future acrolein but also other reactive chemicals the nature of which might be unknown to the persons exposed. This possibility to monitor and potentially to identify unknown adducts is made possible due to the high resolution capability of the Orbitrap instrument, which was purchased with the funds made available from this award.

We have conducted some preliminary experiments to examine the feasibility of this approach.

Chemical modifications of human plasma with 8 reactive chemicals were conducted. Protein precipitates from each reaction were reduced, alkylated with iodoacetamide (IAM), digested with trypsin and then combined. The peptide mixture was separated on a Hypersil GOLD C18 column and analyzed by LTQ Orbitrap XL in data dependent mode.

The calculated m/z values for the MS/MS b and y ion series of the non-adducted HSA T5 fragments are shown in the table below.

Tryptic Peptide T5 of HSA: **ALVLIAFAQYLQQCP**FEDHVK

21 residues and monoisotopic mass: 2432.2562 Da

Calculated m/z of T5 fragments

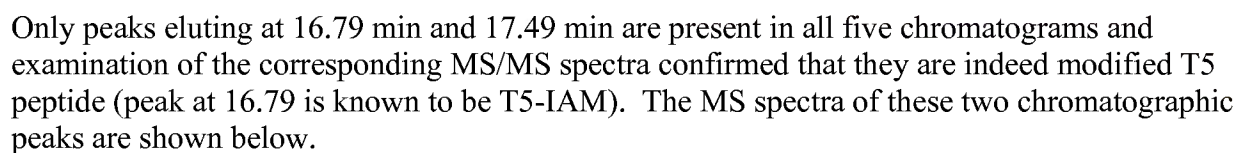
No	Residues	b	Y	
5	I	510.3650	2036.9899	17
6	A	581.4021	1923.9058	16
7	F	728.4705	1852.8687	15
8	A	799.5076	1705.8003	14
9	Q	927.5662	1634.7632	13
10	Y	1090.6295	1506.7046	12
11	L	1203.7136	1343.6412	11
12	Q	1331.7722	1230.5572	10
13	Q	1459.8308	1102.4986	9
14	C	1562.8399	974.4400	8
15	P	1659.8927	871.4308	7
16	F	1806.9611	774.3781	6
17	E	1936.0037	627.3097	5
18	D	2051.0307	498.2671	4
19	H	2188.0896	383.2401	3
20	V	2287.1580	246.1812	2
21	K	-	147.1128	1

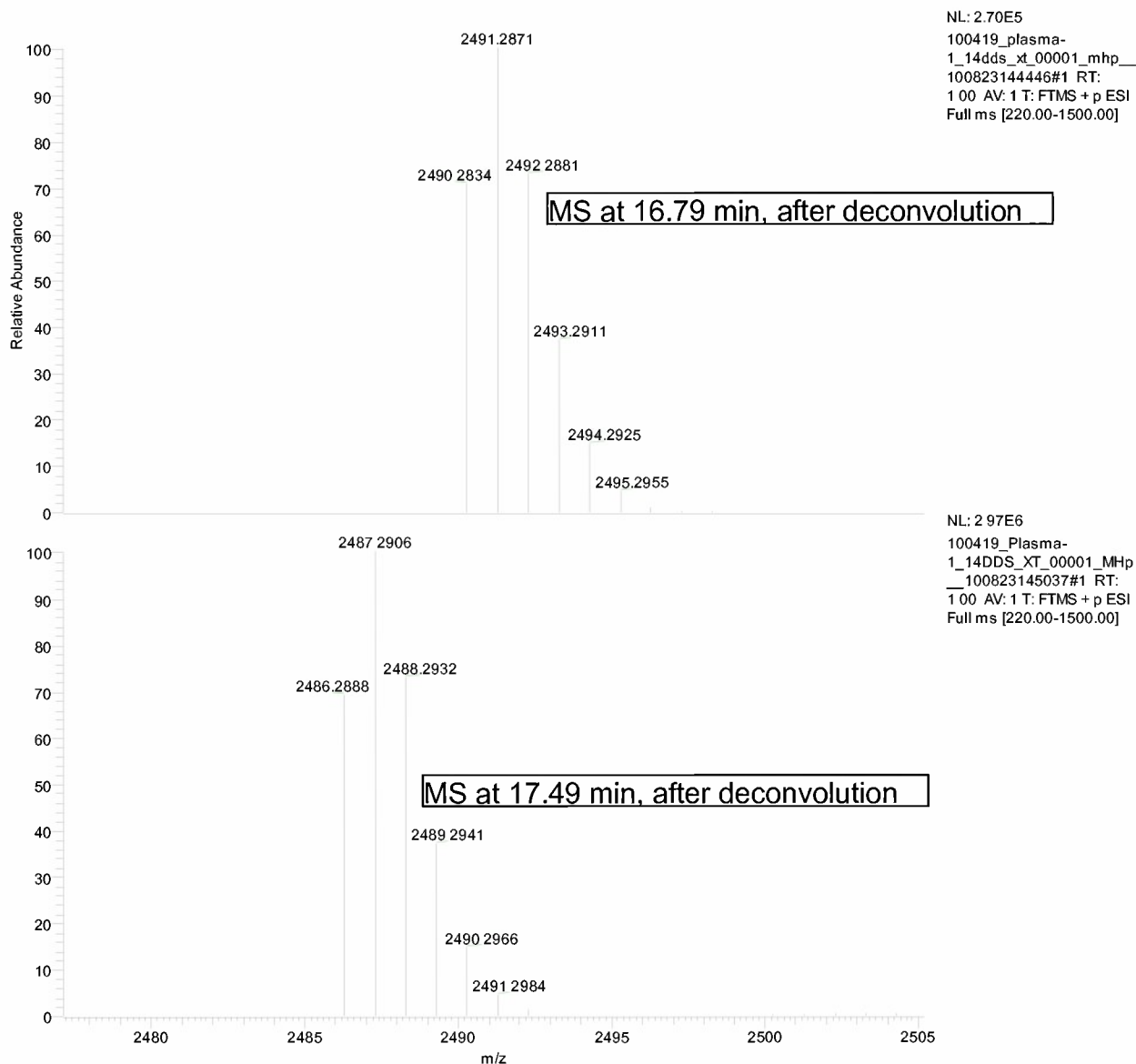
The fragments colored in blue (b_5^+ - b_{13}^+ and y_1^+ - y_7^+) do not undergo a mass shift whether or not C34 is adducted and thus can be used to identify MS/MS spectra that are likely related to the T5 peptide, with or without modification. From the MS/MS spectra of free T5 and T5-IAM, we know b_5^+ , b_6^+ , b_7^+ , b_8^+ , and b_{11}^+ have good abundance in the MS/MS spectra and signals from these fragments can be used to identify modified T5 peptides. The fragments colored in red (b_{14}^+ - b_{20}^+ and y_8^+ - y_{17}^+) do undergo a mass shift on adduct formation and these fragments can then be used to confirm modification at C34 and provide information about the identity of the modification.

In this approach, we can use data dependent scan techniques to acquire as many MS/MS spectra as possible from a trypsin-digested HSA sample. We can then draw ion chromatograms of b_5^+ , b_6^+ , b_7^+ , b_8^+ , and b_{11}^+ of T5, to find MS/MS spectra that are likely related to T5. All T5 related peptides should show up as a peak at same retention time in all five ion chromatograms.

Because of the high resolution capabilities of the Orbitrap mass spectrometer, we are able to draw ion chromatograms with a very narrow window of $m/Z \pm 0.03$ Th, which can significantly decrease false positive identification of MS/MS spectra. After the MS/MS spectra that are likely related to T5 are identified through this process, we can then examine each possible MS/MS spectra to confirm or rule out that the MS/MS spectra are from a modified T5. When the MS/MS spectra are confirmed to come from a modified T5, we can further obtain an accurate mass shift caused by the modification, which can provide the elemental compositions of the

To illustrate this principle, a plasma sample treated only with AN and then processed as described above was analyzed by this method and the data dependent ion chromatograms for this sample are illustrated below.





The top MS spectrum of the chromatographic peak at 16.79 minutes is known from previous MS/MS analysis to be T5, where C34 is blocked by IAM (T5-IAM, data not shown). The second peak at 17.49 is from an “unknown” C34 adduct.

The detected $[M+H]^+$ ions for the chromatographic peaks at 16.79 and 17.49 derived from the above MS spectra are tabulated below.

Rt (min)	Detected $[M+H]^+$	Theoretical/Corrected $[M+H]^+$	Mass Shift
16.79	2490.2834	2490.2850	
17.49	2486.2888	2486.2904	53.0269

The T5-IAM $[M+H]^+$ ion detected at 2490.2834 is 0.0016 Da less than its theoretical value of 2490.2850. If we use the T5-IAM ion as a lock mass, we can then correct the detected $[M+1]^+$ ion at 2486.2888 of the “unknown” to 2486.2904 by adding 0.0016 Da. If we now subtract this m/z value from the theoretical m/z of the $[M+H]^+$ ion of non-adducted T5 (m/z 2433.2635) we can calculate the corrected Mass Shift of the “unknown” adduct as 53.0269. Submitting this mass shift to chemical composition software, using a 5 mDa error window, yields only one chemical composition, namely C_3H_3N , which is consistent with the unknown adduct resulting from acrylonitrile, $CH_2=CHCN$.

In a similar way, seven other “unknown” adducts of HSA-C34 could be detected and identified (data not shown).

Key Research Accomplishments:

- explored several methodologies for assessing exposure to the TIC acrylonitrile
 - GSH (p4-7)
 - Albumin (p7-11)
 - Hemoglobin (p11-15)
- estimated the most sensitive of these biomarkers of exposure
 - GSH (p16-19)
 - Albumin (p19-21)
 - Hemoglobin (p21-24)
- explored procedures for minimizing the biomarker analysis time
 - fast trypsin digestion (p24-30)
 - fast sample analysis (p30-37)
- explored expanding these methodologies for other “unknown” reactive chemicals (p37-41)

Reportable Outcomes:

Poster presented at the Society of Toxicology (SOT) Meeting, Salt Lake City, Utah. March 7-11 (2010), Abstract in Appendix.

Platform Presentation at the 58th ASMS Conference on Mass Spectrometry and Allied Topics, May 23 - 27, 2010, Salt Lake City, Utah, Abstract in Appendix.

Poster presented at the XII International Congress of Toxicology (IUTOX) Barcelona, Spain, July 19-23, 2010, Abstract in Appendix.

Conclusions:

In this project we have developed the methodology for measuring several blood adducts of the Toxic Industrial Chemical, acrylonitrile, for use as biomarkers of exposure. We have identified which of these biomarkers is most sensitive to TIC exposure. We have minimized the analysis time of this biomarker so that the methodology would be applicable for triage of exposed

individuals and we have explored the possibility of expanding this methodology not only to acrolein exposure but also additional reactive chemicals.

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Appendices:

Abstract of Poster presented at the Society of Toxicology (SOT) Meeting, Salt Lake City, Utah, March 7-11 (2010).

Second Order Rate Constants for the In Vitro Reaction of the Toxic Industrial Chemical Acrylonitrile with the Most Reactive Sites in Human Blood.

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The twenty-first century warfighter and civilians will encounter the challenge of the potential use of toxic industrial chemicals (TICs) as chemical warfare agents. Preparedness for acts of chemical terrorism and warfare require prevention, detection and response to such attacks. Appropriate chemical testing will allow for proper diagnosis and treatment of exposed individuals. Acrylonitrile (AN) is a TIC produced in large quantities by the chemical industry and is acutely toxic. Our overall objective is to define the chemical signatures of AN-adducts in

human blood, and to eventually devise a rapid, high throughput screening technology to enable examination of large groups of individuals following a known or suspected exposure. Toward this end, in this project we have measured the second order rate constants for the reaction of AN with the most reactive sites in human blood in vivo. Fresh human blood, red blood cell lysates and plasma were incubated, under pseudo first-order conditions, with 100mM AN at 37C and the disappearance of glutathione (GSH) and the appearance of the AN-adducts of GSH, hemoglobin beta-Cys93 (HbBC93-AN) and albumin Cys34 (AbC34-AN) were monitored by spectrophotometry (spec) and/or high resolution mass spectrometry (MS). The second order rate constants in M-1s-1 were as follows: disappearance of GSH in whole blood, 0.0813 (spec) and 0.0799 (MS); appearance of GS-AN in whole blood, 0.0776 (MS), appearance of HbBC93-AN in rbc lysate, 0.000722 (MS) and appearance of A-C34-AN in plasma, 0.224 (MS). The data indicate that the most reactive site for AN in human blood is Cys-34 of plasma albumin. This site reacts 2.8 times faster than GSH and 310 times faster than HbBC93. Future studies will be directed toward determining the limits of detection of this adduct and minimizing the analysis time. Supported by DOD, U.S. Army Medical Research and Material Command, W81XWH-08-1-0047.

Abstract of a Platform Presentation at the 58th ASMS Conference on Mass Spectrometry and Allied Topics, May 23 - 27, 2010, Salt Lake City, Utah

Human Serum Albumin Cys34 Adducts as a Biomarker for Exposure to Unknown Reactive Chemicals.

Authors: Jian Cai, Frederick W. Benz, Donald E. Nerland, Harrell E. Hurst, William M. Pierce Jr.

Introduction: Some endogenous and exogenous chemicals react with proteins to form adducts that can alter protein function and cause toxic effects. However, protein adducts can also serve as biomarkers of exposure to reactive chemicals. Often the exact chemical involved in the exposure is not known. In this case, identification of the adduct would be difficult. Although specialized MS software is available to perform this task, all possible modifications would need to be examined for each spectrum and thus would be very time consuming. Here we report a method that allows screening of human exposure to unknown reactive chemicals using adducts of human serum albumin cysteine-34 (HSA-C34) as the biomarker.

Method: Chemical modifications of human plasma with 8 reactive chemicals were conducted. Protein precipitates from each reaction were reduced, alkylated with iodoacetamide (IAA), digested with trypsin and then combined. The peptide mixture was separated on a Hypersil GOLD C18 column and analyzed by LTQ Orbitrap XL in data dependent mode. C34 appears in HSA tryptic peptide T5. Ion chromatograms of the fragments of T5 that are not shifted by adduct formation on C34 were drawn with a very narrow m/Z window. Peptides modified at C34 would be observed as a peak in all ion chromatograms. Modifications at C34 were confirmed by MS/MS spectra. The elemental compositions of the adducts were obtained from the mass shifts of precursor ions of the adducts.

Preliminary data: The high resolution Orbitrap instrument allowed drawing of ion chromatograms with very narrow m/z windows. This significantly increased the specificity of the method. Ion chromatograms of b_5^+ , b_6^+ , b_7^+ , b_8^+ , and b_{11}^+ of T5, drawn with a $m/Z \pm 0.03$ Th window, were used to detect unknown adducts. The method identified all 8 adducts in a test sample. The measurement of accurate masses of precursor ions of adducts also provided the precise mass shifts caused by adduct formation. The accuracy of the mass shift measurements can be further increased by using the T5-IAA precursor ion as a lock mass. These were used to identify the elemental composition of the adducts. With this approach, the error of the adduct mass shift measurements (range 53 to 139 Da) was within 3 mDa. In all cases, the correct elemental composition was the only chemically plausible elemental composition among all possible elemental compositions within the 5 mDa error window obtained from the experimentally observed mass shift. For example, an 'unknown' adduct (T5-X) with $[M+H]^+$ 2486.2822 Da was found at 17.62 min and the modification (+53.02 Da) at C34 was confirmed by its MS/MS spectrum. The mass difference between T5-X and T5 was 53.0282 Da after adjustment with T5-IAA peak (2490.2749 Da) as lock mass. Only one elemental composition, C_3H_3N (error: +1.65 mDa), was found within 5 mDa error window. Indeed, the acrylonitrile adduct (C_3H_3N), was one of the components in the mixture. Adducts at 0.5% level were successfully identified and the detection limit can be further improved by optimizing the experimental conditions. This approach can be used to develop methods to screen unknown adducts at specified locations in any protein if the MS/MS spectrum of the peptide containing the reactive residue is detectable.

Novel aspect: A method was developed to screen for unknown protein adducts and to obtain the accurate elemental composition of the adducts.

Support: Supported by DOD, U.S. Army Medical Research and Material Command, W81XWH-08-1-0047.

Abstract of Poster presented at the XII International Congress of Toxicology (IUTOX) Barcelona, Spain, July 19-23, 2010.

P101-014

Acrylonitrile, adduct, mass spectrometry, biomarker, toxic industrial chemicals

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The twenty-first century warfighter and civilians will encounter the challenge of the potential use of toxic industrial chemicals (TICs) as chemical warfare agents. Preparedness for acts of chemical terrorism and warfare require prevention, detection and response to such attacks. Appropriate chemical testing will allow for proper diagnosis and treatment of exposed individuals. Acrylonitrile (AN) is a TIC produced in large quantities by the chemical industry and is acutely toxic. Our overall objective is to define the chemical signatures of AN-adducts in human blood, and to eventually devise a rapid, high throughput screening technology to enable examination of large groups of individuals following a known or suspected exposure. Toward this end, in this project we have measured the second order rate constants for the reaction of AN

with the most reactive sites in human blood in vivo. Fresh human blood, red blood cell lysates and plasma were incubated, under pseudo first-order conditions, with 100mMAN at 37C and the disappearance of glutathione (GSH) and the appearance of the AN-adducts of GSH, hemoglobin beta-Cys93 (HbBC93-AN) and albumin Cys34 (AbC34-AN) were monitored by spectrophotometry (spec) and/or high resolution mass spectrometry (MS). The second order rate constants in M-1s-1 were as follows: disappearance of GSH in whole blood, 0.0813 (spec) and 0.0799 (MS); appearance of GS-AN in whole blood, 0.0776 (MS), appearance of HbBC93-AN in rbc lysate, 0.000722 (MS) and appearance of A-C34-AN in plasma, 0.224 (MS). The data indicate that the most reactive site for AN in human blood is Cys-34 of plasma albumin. This site reacts 2.8 times faster than GSH and 310 times faster than HbBC93. Future studies will be directed toward determining the limits of detection of this adduct and minimizing the analysis time. Supported by DOD, U.S. Army Medical Research and Material Command, W81XWH-08-1-0047.

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Book Chapters:

1. Benz, F.W. and G.C.K. Roberts. The Interaction of Nucleotides with Bovine Pancreatic Ribonuclease, in *Physico-Chemical Properties of Nucleic Acids*. Vol. 3, p. 77-138. Ed. J. Dushesne, Academic Press, 1973.
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Theses:

1. Benz, F.W. Synthesis and Structure Activity Relationships of Hemicholinium-3 Analogs. Master's Thesis. University of Iowa, Iowa City, IA, 1968.
2. Benz, F.W. Stereo- and Bio-chemical Evaluation of a Series of Oxime Reactivators of Diethylphosphoryl-Acetylcholinesterase. Ph.D. Thesis. University of Iowa, Iowa City, IA, 1970.

Computer Programs:

1. Account/Grant Management Program written in the dBASE Language for University of Louisville requisitions, small purchase orders and fund transfers. Used in over 80 departments University-wide 1985-2002.
2. Grading Program written in the dBASE Language for Department of Pharmacology Courses in Medical and Dental Pharmacology, 1989-2000.
3. Church Data Management System written in the dBASE Language for The Church of the Epiphany, Anchorage, KY for Census, Contribution and Volunteer Tracking, 1985-2000.
4. University of Louisville Space Database, assisted programmer Phillip Goff in developing this application for tracking University Space with an emphasis on research tracking, 2004-Present.

Supporting Data:

Figures and Tables were embedded in the text.

List of all personnel receiving pay from the research effort:

Frederick W. Benz, Ph.D., PI: November 20, 2009 – August 2010

William M. Pierce Jr., Ph.D., PI: April 2008 – November 19, 2009

Jian Cai, Ph.D., Co.I.

Donald E. Nerland, Ph.D., Co.I.

Harrell E. Hurst, Ph.D., Co.I.

Kevin Taylor, B.S., Technician

Delano Turner, B.S., Technician